

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner  
US Department of Commerce  
United States Patent and Trademark  
Office, PCT  
2011 South Clark Place Room  
CP2/5C24  
Arlington, VA 22202  
ETATS-UNIS D'AMERIQUE  
in its capacity as elected Office

Date of mailing (day/month/year) 06 February 2001 (06.02.01)	
International application No. PCT/US00/04744	Applicant's or agent's file reference 32212-PCT
International filing date (day/month/year) 25 February 2000 (25.02.00)	Priority date (day/month/year) 26 February 1999 (26.02.99)
Applicant PETERSEN, Bryon, E. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:  
15 September 2000 (15.09.00)

☐ in a notice effecting later election filed with the International Bureau on:  
\_\_\_\_\_

2. The election ☒ was  
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer I. Britel Telephone No.: (41-22) 338.83.38
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## PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

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NOTIFICATION OF RECEIPT OF  
RECORD COPY

(PCT Rule 24.2(a))

To:

BAKER BOTTS L.L.P.

SEIDE, Rochelle, K.  
Baker Botts LLP  
30 Rockefeller Plaza  
New York, NY 10112-0228  
ETATS-UNIS D'AMERIQUE

00 MAY -8 PM12: 01

TO  
RUSCLS  
MEV

Date of mailing (day/month/year) 19 April 2000 (19.04.00)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 32212-PCT	International application No. PCT/US00/04744

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION (for  
all designated States except US)  
PETERSEN, Bryon, E. et al (for US)

International filing date : 25 February 2000 (25.02.00)  
Priority date(s) claimed : 26 February 1999 (26.02.99)  
Date of receipt of the record copy  
by the International Bureau : 03 April 2000 (03.04.00)  
List of designated Offices :

AP : GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW  
EA : AM,AZ,BY,KG,KZ,MD,RU,TJ,TM  
EP : AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE  
OA : BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG  
National : AE,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EE,ES,FI,GB,  
GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,  
MN,MW,MX,NO,NZ,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,  
ZW

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 740.14.35	Authorized officer: I. Britel Telephone No. (41-22) 338.83.38
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## Continuation of Form PCT/IB/301

## NOTIFICATION OF RECEIPT OF RECORD COPY

Date of mailing (day/month/year) 19 April 2000 (19.04.00)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 32212-PCT	International application No. PCT/US00/04744

**ATTENTION**

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- ☒ time limits for entry into the national phase
- ☐ confirmation of precautionary designations
- ☒ requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

## INFORMATION ON TIME LIMITS FOR ENTERING THE NATIONAL PHASE

The applicant is reminded that the "national phase" must be entered before each of the designated Offices indicated in the Notification of Receipt of Record Copy (Form PCT/IB/301) by paying national fees and furnishing translations, as prescribed by the applicable national laws.

The time limit for performing these procedural acts is **20 MONTHS** from the priority date or, for those designated States which the applicant elects in a demand for international preliminary examination or in a later election, **30 MONTHS** from the priority date, provided that the election is made before the expiration of 19 months from the priority date. Some designated (or elected) Offices have fixed time limits which expire even later than 20 or 30 months from the priority date. In other Offices an extension of time or grace period, in some cases upon payment of an additional fee, is available.

In addition to these procedural acts, the applicant may also have to comply with other special requirements applicable in certain Offices. **It is the applicant's responsibility** to ensure that the necessary steps to enter the national phase are taken in a timely fashion. Most designated Offices do not issue reminders to applicants in connection with the entry into the national phase.

For detailed information about the procedural acts to be performed to enter the national phase before each designated Office, the applicable time limits and possible extensions of time or grace periods, and any other requirements, see the relevant Chapters of Volume II of the PCT Applicant's Guide. Information about the requirements for filing a demand for international preliminary examination is set out in Chapter IX of Volume I of the PCT Applicant's Guide.

GR and ES became bound by PCT Chapter II on 7 September 1996 and 6 September 1997, respectively, and may, therefore, be elected in a demand or a later election filed on or after 7 September 1996 and 6 September 1997, respectively, regardless of the filing date of the international application. (See second paragraph above.)

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

## CONFIRMATION OF PRECAUTIONARY DESIGNATIONS

This notification lists only specific designations made under Rule 4.9(a) in the request. It is important to check that these designations are correct. Errors in designations can be corrected where precautionary designations have been made under Rule 4.9(b). The applicant is hereby reminded that any precautionary designations may be confirmed according to Rule 4.9(c) before the expiration of 15 months from the priority date. If it is not confirmed, it will automatically be regarded as withdrawn by the applicant. There will be no reminder and no invitation. Confirmation of a designation consists of the filing of a notice specifying the designated State concerned (with an indication of the kind of protection or treatment desired) and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.

## REQUIREMENTS REGARDING PRIORITY DOCUMENTS

For applicants who have not yet complied with the requirements regarding priority documents, the following is recalled.

Where the priority of an earlier national, regional or international application is claimed, the applicant must submit a copy of the said earlier application, certified by the authority with which it was filed ("the priority document") to the receiving Office (which will transmit it to the International Bureau) or directly to the International Bureau, before the expiration of 16 months from the priority date, provided that any such priority document may still be submitted to the International Bureau before that date of international publication of the international application, in which case that document will be considered to have been received by the International Bureau on the last day of the 16-month time limit (Rule 17.1(a)).

Where the priority document is issued by the receiving Office, the applicant may, instead of submitting the priority document, request the receiving Office to prepare and transmit the priority document to the International Bureau. Such request must be made before the expiration of the 16-month time limit and may be subjected by the receiving Office to the payment of a fee (Rule 17.1(b)).

If the priority document concerned is not submitted to the International Bureau or if the request to the receiving Office to prepare and transmit the priority document has not been made (and the corresponding fee, if any, paid) within the applicable time limit indicated under the preceding paragraphs, any designated State may disregard the priority claim, provided that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity to furnish the priority document within a time limit which is reasonable under the circumstances.

Where several priorities are claimed, the priority date to be considered for the purposes of computing the 16-month time limit is the filing date of the earliest application whose priority is claimed.

## PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING  
SUBMISSION OR TRANSMITTAL  
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

BAKER BOTTS L.L.P.

SEIDE, Rochelle, K.

Baker Botts LLP

30 Rockefeller Plaza

New York, NY 10112-0228

ETATS-UNIS D'AMERIQUE

00 MAY 23 PM 12: 16

TO  
RKS  
ALS  
MEV

Date of mailing (day/month/year) 03 May 2000 (03.05.00)	<b>IMPORTANT NOTIFICATION</b>
Applicant's or agent's file reference 32212-PCT	
International application No. PCT/US00/04744	
International publication date (day/month/year) Not yet published	
International filing date (day/month/year) 25 February 2000 (25.02.00)	
Priority date (day/month/year) 26 February 1999 (26.02.99)	
Applicant UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION et al	

- The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
- This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
- An asterisk(\*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
- The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
26 Febr 1999 (26.02.99)	60/122,250	US	28 Apr 2000 (28.04.00)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland  Facsimile No. (41-22) 740.14.35	Authorized officer  Carlos Naranjo  Telephone No. (41-22) 338.83.38
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**NOTICE INFORMING THE APPLICANT OF THE  
COMMUNICATION OF THE INTERNATIONAL  
APPLICATION TO THE DESIGNATED OFFICES**

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:

SEIDE, Rochelle, K.  
Baker Botts LLP  
30 Rockefeller Plaza  
New York, NY 10112-0228  
ETATS-UNIS D'AMERIQUE

BAKER BOTTS L.L.P.

20 SEP 12 AM 11:46

RKS TO  
CLS  
MCA

Date of mailing (day/month/year) 31 August 2000 (31.08.00)		IMPORTANT NOTICE	
Applicant's or agent's file reference 32212-PCT			
International application No. PCT/US00/04744	International filing date (day/month/year) 25 February 2000 (25.02.00)	Priority date (day/month/year) 26 February 1999 (26.02.99)	
Applicant UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION et al			

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:  
AU, KP, KR, US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:  
AE, AL, AM, AP, AT, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EA, EE, EP, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, OA, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW  
The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on 31 August 2000 (31.08.00) under No. WO 00/50048

**REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)**

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

**REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))**

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

Docketed

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer J. Zahra	For 01/242000 by
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38	

End. in pocket

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# INTERNATIONAL COOPERATION TREATY

PCT/US00/04744  
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## INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

From the INTERNATIONAL BUREAU

To:

SEIDE, Rochelle, K.  
Baker Botts LLP  
30 Rockefeller Plaza  
New York, NY 10112-0228  
ETATS-UNIS D'AMERIQUE

BAKER BOTTS L.L.P.

FEB 13 AM 11:58

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PCT  
US

### IMPORTANT INFORMATION

Date of mailing (day/month/year)

06 February 2001 (06.02.01)

Applicant's or agent's file reference

32212-PCT

International application No.

PCT/US00/04744

International filing date (day/month/year)

25 February 2000 (25.02.00)

Priority date (day/month/year)

26 February 1999 (26.02.99)

Applicant

UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION et al

- The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

AP : GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW

EP : AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE

National : AU, BG, CA, CN, CZ, DE, IL, JP, KP, KR, MN, NO, NZ, PL, RO, RU, SE, SK, US

- The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

EA : AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

OA : BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

National : AE, AL, AM, AT, AZ, BA, BB, BR, BY, CH, CR, CU, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IN, IS, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MW, MX, PT, SD, SG, SI, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW

- The applicant is reminded that he must enter the "national phase" before the expiration of 30 months from the priority date before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing, if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

The entry into the European regional phase is postponed until 31 months from the priority date for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

I. Britel

Telephone No. (41-22) 338.83.38

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The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are chosen by the applicant, the full name or two-letter code of that Authority may be indicated by the applicant on the line.

IPEA/ US

# PCT

## CHAPTER II

### DEMAND

under Article 31 of the Patent Cooperation Treaty:  
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only	
Identification of IPEA	Date of receipt of DEMAND
<b>Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION</b>	
Applicant's or agent's file reference 32212-PCT	
International application No. PCT/US00/04744	International filing date (day/month/year) 25 February 2000 ( 25.02.00 )
(Earliest) Priority date (day/month/year) 26 February 1999 ( 26.02.99 )	
Title of invention BONE MARROW TRANSPLANTATION FOR HEPATIC REGENERATION AND REPAIR	
<b>Box No. II APPLICANT(S)</b>	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)  UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION 200 Gardner Steel Conference Center Pittsburgh, PA 15260 US	Telephone No.:  Facsimile No.:  Teleprinter No.:
State (that is, country) of nationality: US	State (that is, country) of residence: US
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)  PETERSEN, BRYON E. 243 Willow Drive Monroeville, PA 15146 US	
State (that is, country) of nationality: US	State (that is, country) of residence: US
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)  GOFF, JULIE P. 7531 Tuscarora Street Pittsburgh, PA 15208 US	
State (that is, country) of nationality: US	State (that is, country) of residence: US
<input type="checkbox"/> Further applicants are indicated on a continuation sheet.	



**Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The following person is ☒ agent ☐ common representative  
 and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.  
☐ is hereby appointed and any earlier appointment of (an) agent(s) /common representative is hereby revoked.  
☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official  
The address must include postal code and name of country.)*

SEIDE, ROCHELLE K. and  
 STEPHENS, CARMELLA L.  
 Baker Botts LLP  
 30 Rockefeller Plaza  
 New York, NY 10112-0228  
 US

Telephone No.:  
 (212) 705-5000

Facsimile No.:  
 (212) 705-5020

Teleprinter No.:

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

**Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION****Statement concerning amendments:\***

1. The applicant wishes the international preliminary examination to start on the basis of:

☐ the international application as originally filed.

the description ☒ as originally filed

☐ as amended under Article 34

the claims ☒ as originally filed

☐ as amended under Article 19 (together with any accompanying statement)

☐ as amended under Article 34

the drawings ☐ as originally filed

☒ as amended under Article 34

2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

\* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

☒ which is the language in which the international application was filed.

☐ which is the language of a translation furnished for the purposes of international search.

☐ which is the language of publication of the international application.

☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

**Box No. V ELECTION OF STATES**

The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes not to elect:

**Box No. VI CHECK LIST**

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:

- |   |   |           |
|---|---|-----------|
| 1. translation of international application                             | : | sheets    |
| 2. amendments under Article 34  | : | 30 sheets |
| 3. copy (or where required, translation) of amendments under Article 19 | : | sheets    |
| 4. copy (or, where required, translation) of statement under Article 19 | : | sheets    |
| 5. letter   | : | sheets    |
| 6. other ( <i>specify</i> )   | : | sheets    |

For International Preliminary Examining Authority use only

received not received

<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>

The demand is also accompanied by the item(s) marked below:

- |  |   |
|--|---|
| 1. <input checked="" type="checkbox"/> fee calculation sheet                             | 4. <input type="checkbox"/> statement explaining lack of signature                                  |
| 2. <input type="checkbox"/> separate signed power of attorney                            | 5. <input type="checkbox"/> nucleotide and or amino acid sequence listing in computer readable form |
| 3. <input type="checkbox"/> copy of general power of attorney: reference number, if any: | 6. <input checked="" type="checkbox"/> other ( <i>specify</i> ): Transmittal Letter                 |

**Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE**

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

  
 Rochelle K. Seide (Agent)

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

## PCT

## FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No. <b>PCT/US00/04744</b>	For International Preliminary Examining Authority use only
Applicant's or agent's file reference <b>32212-PCT</b>	Date stamp of the IPEA
Applicant <b>UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION</b>	
<b>Calculation of prescribed fees</b>	
1. Preliminary examination fee .....	<b>750.00</b> <span style="border: 1px solid black; padding: 0 5px;">P</span>
2. Handling fee <i>(Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)</i> .....	<b>153.00</b> <span style="border: 1px solid black; padding: 0 5px;">H</span>
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box .....	<div style="border: 1px solid black; padding: 2px; width: 100px; margin: 0 auto;"> <b>903.00</b> </div> <div style="border: 1px solid black; padding: 2px; width: 100px; margin: 0 auto;"> <b>TOTAL</b> </div>
<b>Mode of Payment</b>	
<input type="checkbox"/> authorization to charge deposit account with the IPEA (see below)	<input type="checkbox"/> cash
<input checked="" type="checkbox"/> cheque	<input type="checkbox"/> revenue stamps
<input type="checkbox"/> postal money order	<input type="checkbox"/> coupons
<input type="checkbox"/> bank draft	<input type="checkbox"/> other (specify):
<b>Deposit Account Authorization</b> <i>(this mode of payment may not be available at all IPEAs)</i>	
The IPEA/ <u>US</u> <input type="checkbox"/> is hereby authorized to charge the total fees indicated above to my deposit account.	
<input checked="" type="checkbox"/> <i>(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)</i> is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.	
<b>02-4377</b>	<b>15 September 2000</b>
Deposit Account Number	Date (day/month/year)
Signature	

# PATENT COOPERATION TREATY

32212  
PCT

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

ROCHELLE K. SEIDE  
BAKER BOTTS LLP  
30 ROCKEFELLER PLAZA  
NEW YORK NY 10112-0228

**PCT**

BAKER BOTTS LLP

NOTIFICATION OF RECEIPT  
OF DEMAND BY COMPETENT INTERNATIONAL  
PRELIMINARY EXAMINING AUTHORITY

(PCT Rule 59.3(e) and 61.1(b), first sentence  
and Administrative Instructions, Section 601(a))

Date of mailing  
(day/month/year)

01 DEC 2000

Applicant's or agent's file reference

32212-PCT

**IMPORTANT NOTIFICATION**

International application No.

PCT/US00/04744

International filing date (day/month/year)

25 FEB 00

Priority date (day/month/year)

26 FEB 99

Applicant

UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH  
SYSTEM OF HIGHER EDUCATION

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

**IPEA/US 15 SEP 2000**

2. That date of receipt is:

- ☒ the actual date of receipt of the demand by this Authority (Rule 61.1(b)).  
☐ the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).  
☐ the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. ☐ **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

- ☐ (If applicable) This notification confirms the information given by telephone, facsimile transmission or in person on:

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

8/26/01

Name and mailing address of the IPEA/US

Assistant Commissioner for Patents  
Box PCT  
Washington, D.C. 20231  
Facsimile No.

Attn: IPEA/US

Authorized officer

LARRY HAMMOND  
Telephone No.

# PATENT COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: ROCHELLE K. SEIDE  
BAKER BOTTS LLP  
30 ROCKEFELLER PLAZA  
NEW YORK, NY 10112-0228

**PCT**

BAKER BOTTS L.L.P.

01 MAY 29 PM 3: 38

WRITTEN OPINION TO

(PCT Rule 66)

Date of Mailing  
(day/month/year)

**21 MAY 2001**

Applicant's or agent's file reference

32212-PCT

REPLY DUE

within ONE months  
from the above date of mailing

International application No.

PCT/US00/04744

International filing date (day/month/year)

25 FEBRUARY 2000

Priority date (day/month/year)

26 FEBRUARY 1999

International Patent Classification (IPC) or both national classification and IPC  
Please See Supplemental Sheet.

Applicant

UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Docketed

For 6/21/2001 By *[Signature]*

3. The applicant is hereby invited to reply to this opinion.

**When?** See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).~~

**How?** By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

**Also** For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis. For an informal communication with the examiner, see Rule 66.6.

**If no reply is filed,** the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 26 JUNE 2001

Name and mailing address of the IPEA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

*Quang Nguyen*  
QUANG NGUYEN, Ph.D.

Telephone No. (703) 308-0196

*See in Packet*

## I. Basis of the opinion

## 1. With regard to the elements of the international application:\*

☐ the international application as originally filed☒ the description:

pages 1-34, as originally filed  
pages NONE, filed with the demand  
pages NONE, filed with the letter of

☒ the claims:

pages 35-37, as originally filed  
pages NONE, as amended (together with any statement) under Article 19  
pages NONE, filed with the demand  
pages NONE, filed with the letter of

☒ the drawings:

pages NONE, as originally filed  
pages 1-28, filed with the demand  
pages NONE, filed with the letter of

☒ the sequence listing part of the description:

pages NONE, as originally filed  
pages NONE, filed with the demand  
pages NONE, filed with the letter of

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.  
These elements were available or furnished to this Authority in the following language which is:☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).☐ the language of publication of the international application (under Rule 48.3(b)).☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

## 3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

☐ contained in the international application in printed form.☐ filed together with the international application in computer readable form.☐ furnished subsequently to this Authority in written form.☐ furnished subsequently to this Authority in computer readable form.☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.4. ☒ The amendments have resulted in the cancellation of:☒ the description, pages NONE☒ the claims, Nos. NONE☒ the drawings, sheets/fig. NONE5. ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

\* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".

WRITTEN OPINION

International application No.

PCT/US00/04744

**V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. statement

Novelty (N)

Claims 1-19 YES  
Claims 20-21 NO

Inventive Step (IS)

Claims None YES  
Claims 20-24 NO

Industrial Applicability (IA)

Claims 1-24 YES  
Claims NONE NO

2. citations and explanations

Claims 20 and 21 lack novelty under PCT Article 33(2) as being anticipated by Petersen et al. (FASEB J. 12:A468, 1998) or Petersen et al. (Hepatology 27:433-445, 1998) or Craig et al. (J. Exp. Med. 177:1331-13342, 1993).

The claims are drawn to a method for enriching for oval cells comprising disaggregating liver tissue to form a single cell suspension of hepatic cells and purification from the single cell suspension of Thy1.1 positive hepatic cells from Thy1.1 negative hepatic cells, wherein the Thy1.1 positive cells comprise the oval cells; the same method wherein the Thy1.1 positive hepatic cells are purified from Thy1.1 negative cells using a Thy-1 specific antibody.

Both Petersen et al. (FASEB J.) and Petersen et al. (Hepatology) teach a method for isolating a highly enriched population of hepatic oval cells expressing the hematopoietic stem cell marker Thy-1 using flow cytometry coupled with the use of a Thy-1 antibody (See the abstract for Petersen et al. in FASEB J., and page 434, col. 2 in Petersen et al. in Hepatology).

Craig et al. disclose a method for isolating human hematopoietic progenitor cells expressing Thy-1 from human fetal liver using flow cytometry coupled with the use of a novel anti-Thy-1 antibody, 5E10 (See page 1332, sections titled cells and flow cytometry). The method disclosed by Craig et al. is indistinguishable from the instant claimed method, and therefore the isolated Thy1, CD34+ hematopoietic progenitor cell population from human fetal liver comprises the oval cells.

Therefore, these references clearly anticipate the instantly claimed invention.

Claims 22-24 lack an inventive step under PCT Article 33(3) as being obvious over Petersen et al. (FASEB J. 12:A468, 1998) or Petersen et al. (Hepatology 27:433-445, 1998) or Craig et al. (J. Exp. Med. 177:1331-13342, 1993) in view of Reid et al. (U.S. Patent 5,789,246).

The claims are drawn to a composition comprising an enriched population of oval cells in a physiologically acceptable (Continued on Supplemental Sheet.)

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because of the reasons discussed below.

Claims 1-5 and 11-13 are drawn to a method for stimulating liver regeneration in a subject having a liver disorder comprising administering of bone marrow cells to said subject in an amount sufficient to result in the production of hepatocytes, bile ductal cells and/or oval cells; the same method wherein the bone marrow cells are injected or transplanted into the liver; the same wherein the bone marrow are genetically engineered to express a functional active protein or wherein the bone marrow cells are on a support matrix or wherein the bone marrow cells are contacted with a growth factor prior to administration.

Claims 6-12 and 14 are drawn to a method for stimulating liver regeneration in a subject having a liver disorder comprising the administration of enriched oval cells to a subject in an amount sufficient to result in the production of hepatocytes, bile ductal cells and/or oval cells; the same method wherein the oval cells are injected or transplanted into the liver; the same wherein the oval cells are genetically engineered to express a functionally active protein or wherein the oval cells are on a support matrix or wherein the oval cells are contacted with a growth factor prior to administration.

Claims 15-19 are directed to a method for stimulating pancreatic regeneration in a subject having a pancreatic disorder comprising administering of bone marrow cells to said subject in an amount sufficient to result in the production of pancreatic cells; the same method wherein the bone marrow cells are injected or transplanted into the pancreas; or the same wherein the bone marrow cells are genetically engineered to express a functionally active protein or wherein the bone marrow cells are on a support matrix.

The instant application teaches by exemplification demonstrating that oval cells and mature hepatocytes were derived from a cell population originating from the bone marrow using three different approaches: (1) bone marrow transplantation from male rats into lethally irradiated syngeneic females and detection of donor cells in the recipients by means of DNA probes to the Y chromosome sry region, (2) bone marrow transplantation from DPPIV-positive male rats into DPPIV-negative syngeneic females and detection of the DPPIV-positive cells in the recipient animals, and (3) total liver transplantation for Lewis rats that express L21-6 antigen as recipients with Brown-Norway rats that do not express this antigen as allogeneic donors; in conjunction with the 2-acetyl amino fluorene (2-AAF) and carbon tetrachloride (CCl<sub>4</sub>) or partially hepatectomy protocols to induce oval cell activation and proliferation.

(Continued on Supplemental Sheet.)



**Supplemental Box**

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

**TIME LIMIT:**

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

**CLASSIFICATION:**

The International Patent Classification (IPC) and/or the National classification are as listed below:  
IPC(7): A61K 35/28, 35/407; A61P 1/16, 1/18; C12N 5/06, 5/08 and US Cl.: 424/93.1, 93.2, 93.21; 435/1.1, 320.1, 455, 325, 363, 370, 372, 375

**V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):**

carrier, a composition of matter comprising an enriched population of oval cells attached to a matrix and the same wherein said composition of matter further comprising a growth factor associated with the matrix.

Both Petersen et al. (FASEB J.) and Petersen et al. (Hepatology) disclose a composition comprising highly enriched hepatic oval cells expressing the hematopoietic stem cell marker Thy-1 (See the abstract for Petersen et al. in FASEB J., and page 434, col. 2 in Petersen et al. in Hepatology). Craig et al. also teach an isolated Thy1, CD34+ hematopoietic progenitor cell population from human fetal liver, most likely enriched with oval cells (See page 1332, sections titled cells and flow cytometry). However, none of the references teach the disclosed compositions in a physiologically acceptable carrier, or wherein the oval cells are attached to a matrix, preferably in the presence of a growth factor associated with the matrix.

Reid et al. disclose a composition comprising a cell culture of immature animal cells, including liver, pancreas, gut, lung or bone marrow cells which contain at least a population of hepatocyte precursor cells (oval cells) capable of differentiating into hepatocytes, basal serum-free culture medium (physiologically acceptable carrier), extracellular matrix (collagen, fibronectin, laminin and others) and liver stromal cells (See claim 1, col. 2, lines 6-67 and col. 3, lines 1-7). Reid et al. further teach that growth factors such as interleukins, fibroblast growth factors, growth hormones, transforming growth factors and others may be included in said cell culture. Under such cell culture conditions, growth factors can be associated with the extracellular matrix. Additionally, co-cultured liver stromal cells also secrete growth factors that bind to their pericellular matrix.

Accordingly, at the time the instant invention was made one of ordinary skilled artisan would have been motivated to modify the compositions disclosed by Petersen et al. and Craig et al. by culturing enriched hepatic oval cells expressing Thy-1 under conditions taught by Reid et al. One of ordinary skilled in the art would have been motivated to carry out the above modification to expand and proliferate the immature oval cell population for various studies of liver cells, such as toxicology or pharmacology studies or carcinogenic studies as suggested by Reid et al. (See abstract and col. 7, lines 52-60). Therefore, the instant claims are obvious over Petersen et al. or Craig et al. in view of Reid et al.

Claims 1-19 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest methods for stimulating liver regeneration or pancreatic regeneration in a subject having a liver disorder or a pancreatic disorder, respectively, using bone marrow cells or enriched oval cells as claimed.

**----- NEW CITATIONS -----**

CRAIG et al. Expression of Thy-1 on human hematopoietic progenitor cells. J. Exp. Med. 01 May 1993, Vol. 177, pages 1331-1342, see abstract and page 1332.

SHAFRITZ D.A. Rat liver stem cells: Prospects for the future. Hepatology. 01 December 2000, Vol. 32, pages 1399-1400, see the entire document.

DABEVA et al. Proliferation and differentiation of fetal liver epithelial progenitor cells after transplantation into adult rat liver. Am. J. Pathol. 01 June 2000, Vol. 156, pages 2017-2031, see the entire document.

DANG et al. Gene therapy and translational cancer research. Clin. Cancer Res. 01 February 1999, Vol. 5, pages 471-474, see the entire document.

RIDDELL et al. T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. Nature Med. 01 February 1996, Vol. 2, pages 216-223, see the entire document.

**Supplemental Box**

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 11

**VIII. CERTAIN OBSERVATIONS ON THE APPLICATION (Continued):**

The evidence has been noted and considered. However, the evidence can not be extrapolated to the instant claimed invention. When read in light of the disclosure, the instant claims are drawn to methods for treating a subject having a liver disorder (e.g. cirrhosis of liver, alcohol induced hepatitis, chronic hepatitis, alpha<sub>1</sub>-antitrypsin) or a pancreatic disorder using any and all bone marrow cells or any and all enriched oval cells including those that are genetically modified to express any and all functional active proteins such as growth factors, cytokines, hormones among others.

The instant disclosure is not enabled for such a broadly claimed invention because it fails to provide sufficient guidance demonstrating that any therapeutic effects has been achieved for a patient having a liver disorder or a pancreatic disorder by administering bone marrow cells or enriched oval cells into said patient. The mere detection of oval cells and mature hepatocytes possessing the Y chromosomal marker or expressing DPP-IV or L21-6 antigen in the disclosed rat models is not deemed to be equivalent or correlated with the therapeutic effects or results contemplated by the instant claimed invention. This is because the biological significance of these oval cells and mature hepatocytes for treating patients having a hepatic or pancreatic disorder is uncertain and that the physiological art is known to be unpredictable. Moreover, even in a recent review on liver stem cells (Hepatology 32:1399-1400, 2000), Shafritz stated that "...liver transplantation is the only available current therapy for end-stage liver failure....finding alternative methods for liver replacement is of utmost importance. One such method would be functional repopulation of the diseased liver by cell transplantation" (page 1399, col. 1, lines 1-6). Thus, it appears that methods for treating a patient suffering from any hepatic or pancreatic disorder using cell transplantation, for this instance bone marrow cells or oval cells, is not achievable or routine or predictable in the year 2000, let alone at the effective filing date of the present application. Accordingly, in the absence of guidance provided by the instant disclosure, it would have required undue experimentation without a predictable expectation of success for one skilled in the art to make and use the claimed invention.

The instant claims encompass the use of any and all bone marrow cells or oval cells, encompassing those derived from autologous, allogeneic and xenogeneic donors. It is well known in the art that the transplantation of allogeneic and xenogeneic cells into a patient will elicit adverse host immune responses resulting in the rejection of the transplanted cells. The instant disclosure fails to provide sufficient guidance regarding how such adverse host's immune reactions would be bypassed so that any and all allogeneic or xenogeneic bone marrow cells or oval cells would be stably engrafted and properly differentiated into hepatocytes in a treated patient for a sufficient period of time to yield any therapeutic results contemplated by the claimed invention. In the absence of sufficient teachings provided by the present disclosure, it would have required undue experimentation for a skilled artisan to make and use the broadly claimed invention.

The instant claims encompass the differentiation of administered bone marrow cells or oval cells into hepatocytes or bile ductal cells. However, there is no evidence in the present disclosure indicating that the bone marrow cells or oval cells can actually differentiate *in vivo* into bile ductal cells under any conditions. Furthermore, the bipotent nature of liver stem cells or progenitor cells is still uncertain as noted by Shafritz "the clonality and bipotent nature of isolated liver stem/progenitor cells need to be confirmed by *in vivo* transplantation studies" (page 1400, col. 1, middle paragraph). In the absence of such guidance provided by the instant disclosure, it would again require undue experimentation for one skilled in the art to make and use the broadly claimed invention.

The instant broad claims encompass any and all sites for administering bone marrow cells or oval cells into a patient. However, Dabeva et al. (Am. J. Pathol. 156:2017-2031, 2000) have noted that "Several studies also report successful engraftment and differentiation of early fetal liver tissue or cell suspensions after transplantation into ectopic sites. However, engrafted liver tissue masses at ectopic sites do not expand very much, and it is unlikely that such limited liver transplantation will have broad therapeutic application" (page 2029, col. 2, last three lines continue to line 4 in col. 1 of page 2030). With respect to the above teaching of Dabeva et al., coupled with the lack of sufficient guidance provided by the instant disclosure, it would again require undue experimentation for a skilled artisan to administer bone marrow cells or oval cells into any and all sites in a patient for achieving the desired therapeutic effects contemplated by the present application.

The instant claims encompass the use of genetically modified bone marrow cells and oval cells. Therefore, the nature of an embodiment of the instant claims falls within the realm of gene therapy (*ex vivo* gene therapy) which at the effective filing date of the present application was immature and highly unpredictable. At about the effective filing date of the instant invention, Dang et al. (Clin. Cancer Res. 5:471-474, 1999) noted that "This workshop reviewed some recent advances in gene delivery, gene expression, immune manipulation, and the development of molecular targets and stressed that all of these fields will need further advancement to make gene therapy a reality" (page 471, col. 1, last sentence of first paragraph). There are several factors known to limit the effectiveness of gene therapy, among which are the lack of optimal vectors, the lack of a long-term and stable transgene expression *in vivo*. With respect to the breadth of the instant claims encompassing the expression of any and all functional active proteins, factors such as the level of mRNA produced, the stability of the protein or polypeptide produced, the protein's proper compartmentalization within the cell or its secretory fate differ dramatically based on which protein or polypeptide being produced, and therefore the desired therapeutic effects sought to achieve. Thus, the level of transgene expression *in vivo*, its duration, and its *in vivo* therapeutic effects can not be predictable, nor these obstacles can be overcome with routine experimentation. With the lack of guidance provided by the instant disclosure regarding to the above issues and in light of the unpredictable nature of the gene therapy art, it would have required undue experimentation without a predictable expectation of success for one skilled in the art

**Supplemental Box**

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 12.

to make and use the claimed invention.

Finally, it is also unclear whether the transplanted genetically modified bone marrow cells or oval cells could be stably engrafted and differentiated properly into the treated patient. It should be noted that genetically modified cells may express gene products associated with the recombinant vectors used to deliver the desired transgene, e.g. adenoviral functional proteins. These and some of the transgene products themselves would also be recognized by the host immune system as foreign proteins and adverse immune reactions would be mounted against the transplanted genetically modified cells. As an example, Riddell et al. (Nature Med. 2:216-223, 1996) reported that five out of six patients seropositive for human immunodeficiency virus developed cytotoxic T-lymphocytes responses specific to a novel protein and eliminated infused autologous CD8+ HIV-specific cytotoxic T cells transduced with a suicide gene encoding a hygromycin phosphotransferase and the herpes virus thymidine kinase fusion protein. The instant disclosure fails to provide any guidance demonstrating that genetically modified bone marrow cells or oval cells would be able to avoid adverse host immune rejection reactions upon transplantation into a patient in need of, such that they could be stably engrafted, proliferate, differentiate and express effective levels of desired transgene products to yield any therapeutic effects contemplated by the instant invention. In the absence of such guidance, it would again required undue experimentation without a predictable expectation of success for a skilled artisan to make and use the claimed invention.

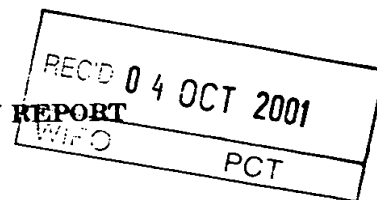
Claims 1-19 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)



Applicant's or agent's file reference 52212-PCT	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/116)	
International application No. PCT/US00/04744	International filing date (day/month/year) 25 FEBRUARY 2000	Priority date (day/month/year) 26 FEBRUARY 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 7 sheets.  
☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).  
 These annexes consist of a total of 88 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability, citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand <u>15</u> SEPTEMBER 2000	Date of completion of this report 10 AUGUST 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer  QUANG NGUYEN, PH.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/04744

## I. Basis of the report

## 1. With regard to the elements of the international application:\*

☐ the international application as originally filed☒ the description:

pages 1-34 , as originally filed  
pages NONE , filed with the demand  
pages NONE , filed with the letter of \_\_\_\_\_

☒ the claims:

pages 35-37 , as originally filed  
pages NONE , as amended (together with any statement) under Article 19  
pages NONE , filed with the demand  
pages NONE , filed with the letter of \_\_\_\_\_

☒ the drawings:

pages NONE , as originally filed  
pages 1-28 , filed with the demand  
pages NONE , filed with the letter of \_\_\_\_\_

☒ the sequence listing part of the description:

pages NONE , as originally filed  
pages NONE , filed with the demand  
pages NONE , filed with the letter of \_\_\_\_\_

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.  
These elements were available or furnished to this Authority in the following language \_\_\_\_\_ which is:☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).☐ the language of publication of the international application (under Rule 48.3(b)).☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

## 3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.☐ filed together with the international application in computer readable form.☐ furnished subsequently to this Authority in written form.☐ furnished subsequently to this Authority in computer readable form.☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.4. ☒ The amendments have resulted in the cancellation of:☒ the description, pages NONE☒ the claims, Nos. NONE☒ the drawings, sheets/fig NONE5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).\*\*

\* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

\*\*Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/04744

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement****1. statement**

Novelty (N)	Claims	<u>1-19 and 22-24</u>	YES
	Claims	<u>20-21</u>	NO
Inventive Step (IS)	Claims	<u>1-19</u>	YES
	Claims	<u>20-24</u>	NO
Industrial Applicability (IA)	Claims	<u>1-24</u>	YES
	Claims	<u>NONE</u>	NO

**2. citations and explanations (Rule 70.7)**

Claims 20 and 21 lack novelty under PCT Article 33(2) as being anticipated by Petersen et al. (FASEB J. 12:A468, 1998) or Petersen et al. (Hepatology 27:433-445, 1998).

The claims are drawn to a method for enriching for oval cells comprising disaggregating liver tissue to form a single cell suspension of hepatic cells and purification from the single cell suspension of Thy1.1 positive hepatic cells from Thy1.1 negative hepatic cells, wherein the Thy1.1 positive cells comprise the oval cells; the same method wherein the Thy1.1 positive hepatic cells are purified from Thy1.1 negative cells using a Thy-1 specific antibody.

Both Petersen et al. (FASEB J.) and Petersen et al. (Hepatology) teach a method for isolating a highly enriched population of hepatic oval cells expressing the hematopoietic stem cell marker Thy-1 using flow cytometry coupled with the use of a Thy-1 antibody (See the abstract for Petersen et al. in FASEB J., and page 434, col. 2 in Petersen et al. in Hepatology).

Therefore, these references anticipate the instantly claimed invention.

**Response to Arguments**

With respect to the Peterson references, Applicants stated that they can successfully antedate the cited references upon submission of a Katz type Declaration. Since the Declaration has not been submitted, the lack of novelty for claims 20 and 21 is maintained for the reasons set forth above.

Claims 22-24 lack an inventive step under PCT Article 33(3) as being obvious over Petersen et al. (FASEB J. 12:A468, 1998) or Petersen et al. (Hepatology 27:433-445, 1998) in view of Reid et al. (U.S. Patent 5,789,246).

The claims are drawn to a composition comprising an enriched population of oval cells in a physiologically acceptable carrier, a composition of matter comprising an enriched population of oval cells attached to a matrix and the same wherein said composition of matter further comprising a growth factor associated with the matrix.

(Continued on Supplemental Sheet.)

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/04744

## VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because of the reasons discussed below.

Claims 1-5 and 11-13 are drawn to a method for stimulating liver regeneration in a subject having a liver disorder comprising administering of bone marrow cells to said subject in an amount sufficient to result in the production of hepatocytes, bile ductal cells and/or oval cells; the same method wherein the bone marrow cells are injected or transplanted into the liver; the same wherein the bone marrow are genetically engineered to express a functional active protein or wherein the bone marrow cells are on a support matrix or wherein the bone marrow cells are contacted with a growth factor prior to administration.

Claims 6-12 and 14 are drawn to a method for stimulating liver regeneration in a subject having a liver disorder comprising the administration of enriched oval cells to a subject in an amount sufficient to result in the production of hepatocytes, bile ductal cells and/or oval cells; the same method wherein the oval cells are injected or transplanted into the liver; the same wherein the oval cells are genetically engineered to express a functionally active protein or wherein the oval cells are on a support matrix or wherein the oval cells are contacted with a growth factor prior to administration.

Claims 15-19 are directed to a method for stimulating pancreatic regeneration in a subject having a pancreatic disorder comprising administering of bone marrow cells to said subject in an amount sufficient to result in the production of pancreatic cells; the same method wherein the bone marrow cells are injected or transplanted into the pancreas; or the same wherein the bone marrow cells are genetically engineered to express a functionally active protein or wherein the bone marrow cells are on a support matrix.

The instant application teaches by exemplification demonstrating that oval cells and mature hepatocytes were derived from a cell population originating from the bone marrow using three different approaches: (1) bone marrow transplantation from male rats into lethally irradiated syngeneic females and detection of donor cells in the recipients by means of DNA probes to the Y chromosome *sry* region, (2) bone marrow transplantation from DPPIV-positive male rats into DPPIV-negative syngeneic females and detection of the DPPIV-positive cells in the recipient animals, and (3) total liver transplantation for Lewis rats that express L21-6 antigen as recipients with Brown-Norway rats that do not express this antigen as allogeneic donors; in conjunction with the 2-acetyl aminofluorene (2-AAF) and carbon tetrachloride (CCl<sub>4</sub>) or partially hepatectomy protocols to induce oval cell activation and proliferation.

The evidence has been noted and considered. However, the (Continued on Supplemental Sheet.)

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/04744

## Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

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Continuation of: Boxes I - VIII

### CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:  
IPC(7): A61K 35/28, 35/407; A61P 1/16, 1/18; C12N 5/06, 5/08 and US Cl.: 424/93.1, 93.2, 93.21; 435/1.1, 320.1, 455, 325, 363, 370, 372, 375

### V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

Both Petersen et al. (FASEB J.) and Petersen et al. (Hepatology) disclose a composition comprising highly enriched hepatic oval cells expressing the hematopoietic stem cell marker Thy-1 (See the abstract for Petersen et al. in FASEB J., and page 434, col. 2 in Petersen et al. in Hepatology). However, none of the references teaches the disclosed compositions in a physiologically acceptable carrier, or wherein the oval cells are attached to a matrix, preferably in the presence of a growth factor associated with the matrix.

Reid et al. disclose a composition comprising a cell culture of immature animal cells, including liver, pancreas, gut, lung or bone marrow cells which contain at least a population of hepatocyte precursor cells capable of differentiating into hepatocytes, basal serum-free culture medium (physiologically acceptable carrier), extracellular matrix (collagen, fibronectin, laminin and others) and liver stromal cells (See claim 1, col. 2, lines 6-67 and col. 3, lines 1-7). Reid et al. further teach that growth factors such as interleukins, fibroblast growth factors, growth hormones, transforming growth factors and others may be included in said cell culture. Under such cell culture conditions, growth factors can be associated with the extracellular matrix. Additionally, co-cultured liver stromal cells also secrete growth factors that bind to their pericellular matrix.

Accordingly, at the time the instant invention was made one of ordinary skilled artisan would have been motivated to modify the compositions disclosed by Petersen et al. by culturing enriched hepatic oval cells expressing Thy-1 under conditions taught by Reid et al. One of ordinary skilled in the art would have been motivated to carry out the above modification to expand and proliferate the immature oval cell population for various studies of liver cells, such as toxicology or pharmacology studies or carcinogenic studies as suggested by Reid et al. (See abstract and col. 7, lines 52-60). Therefore, the instant claims are obvious over Petersen et al. in view of Reid et al.

### Response to Arguments

Applicants argued that the Peterson references can not be used as prior art against the present invention because the references were published within one year of the effective filing date of the present application. As indicated above, since the Katz type Declaration has not been submitted, claims 20 and 21 lacks an inventive step under PCT Article 33(3) for the reasons set forth above.

Claims 1-19 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest methods for stimulating liver regeneration or pancreatic regeneration in a subject having a liver disorder or a pancreatic disorder, respectively, using bone marrow cells or enriched oval cells as claimed.

### NEW CITATIONS

CRAIG et al. Expression of Thy-1 on human hematopoietic progenitor cells. J. Exp. Med. 01 May 1993, Vol. 177, pages 1331-1342, see abstract and page 1332.

SHAFRITZ D.A. Rat liver stem cells: Prospects for the future. Hepatology. 01 December 2000, Vol. 32, pages 1399-1400, see the entire document.

DABEVA et al. Proliferation and differentiation of fetal liver epithelial progenitor cells after transplantation into adult rat liver. Am. J. Pathol. 01 June 2000, Vol. 156, pages 2017-2031, see the entire document.

DANG et al. Gene therapy and translational cancer research. Clin. Cancer Res. 01 February 1999, Vol. 5, pages 471-474, see the entire document.

RIDDELL et al. T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. Nature Med. 01 February 1996, Vol. 2, pages 216-223, see the entire document.

### VIII. CERTAIN OBSERVATIONS ON THE APPLICATION (Continued):



## Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

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evidence can not be reasonably extrapolated to the instant claimed invention. When read in light of the disclosure, the instant claims are drawn to methods for treating a subject having a liver disorder (e.g. cirrhosis of liver, alcohol induced hepatitis, chronic hepatitis, alpha<sub>1</sub>-antitrypsin) or a pancreatic disorder using any and all bone marrow cells or any and all enriched oval cells including those that are genetically modified to express any and all functional active proteins such as growth factors, cytokines, hormones among others.

The instant disclosure is not enabled for such a broadly claimed invention because it fails to provide sufficient guidance demonstrating that any therapeutic effects has been achieved for a patient having a liver disorder or a pancreatic disorder by administering bone marrow cells or enriched oval cells into said patient. The mere detection of oval cells and mature hepatocytes possessing the Y chromosomal-marker or expressing DPPIV-or L21-6 antigen in the disclosed rat models is not deemed to be equivalent or correlated with the therapeutic effects or results contemplated by the instant claimed invention. This is because the biological significance of these oval cells and mature hepatocytes for treating patients having a hepatic or pancreatic disorder is uncertain and that the physiological art is known to be unpredictable. Moreover, even in a recent review on liver stem cells (Hepatology 32:1399-1400, 2000), Shafritz stated that "...liver transplantation is the only available current therapy for end-stage liver failure....finding alternative methods for liver replacement is of utmost importance. One such method would be functional repopulation of the diseased liver by cell transplantation" (page 1399, col. 1, lines 1-6). Thus, it appears that methods for treating a patient suffering from any hepatic or pancreatic disorder using cell transplantation, for this instance bone marrow cells or oval cells, is not achievable or routine or predictable in the year 2000, let alone at the effective filing date of the present application. Accordingly, in the absence of guidance provided by the instant disclosure, it would have required undue experimentation without a predictable expectation of success for one skilled in the art to make and use the claimed invention.

The instant claims encompass the use of any and all bone marrow cells or oval cells, encompassing those derived from autologous, allogeneic and xenogeneic donors. It is well known in the art that the transplantation of allogeneic and xenogeneic cells into a patient will elicit adverse host immune responses resulting in the rejection of the transplanted cells. The instant disclosure fails to provide sufficient guidance regarding how such adverse host's immune reactions would be bypassed so that any and all allogeneic or xenogeneic bone marrow cells or oval cells would be stably engrafted and properly differentiated into hepatocytes in a treated patient for a sufficient period of time to yield any therapeutic results contemplated by the claimed invention. In the absence of sufficient teachings provided by the present disclosure, it would have required undue experimentation for a skilled artisan to make and use the broadly claimed invention.

The instant claims encompass the differentiation of administered bone marrow cells or oval cells into hepatocytes or bile ductal cells. However, there is no evidence in the present disclosure indicating that the bone marrow cells or oval cells can actually differentiate *in vivo* into bile ductal cells under any conditions. Furthermore, the bipotent nature of liver stem cells or progenitor cells is still uncertain as noted by Shafritz "the clonality and bipotent nature of isolated liver stem/progenitor cells need to be confirmed by *in vivo* transplantation studies" (page 1400, col. 1, middle paragraph). In the absence of such guidance provided by the instant disclosure, it would again require undue experimentation for one skilled in the art to make and use the broadly claimed invention.

The instant broad claims encompass any and all sites for administering bone marrow cells or oval cells into a patient. However, Dabeva et al. (Am. J. Pathol. 156:2017-2031, 2000) have noted that "Several studies also report successful engraftment and differentiation of early fetal liver tissue or cell suspensions after transplantation into ectopic sites. However, engrafted liver tissue masses at ectopic sites do not expand very much, and it is unlikely that such limited liver transplantation will have broad therapeutic application" (page 2029, col. 2, last three lines continue to line 4 in col. 1 of page 2030). With respect to the above teaching of Dabeva et al., coupled with the lack of sufficient guidance provided by the instant disclosure, it would again require undue experimentation for a skilled artisan to administer bone marrow cells or oval cells into any and all sites in a patient for achieving the desired therapeutic effects contemplated by the present application.

The instant claims encompass the use of genetically modified bone marrow cells and oval cells. Therefore, the nature of an embodiment of the instant claims falls within the realm of gene therapy (*ex vivo* gene therapy) which at the effective filing date of the present application was immature and highly unpredictable. At about the effective filing date of the instant invention, Dang et al. (Clin. Cancer Res. 5:471-474, 1999) noted that "This workshop reviewed some recent advances in gene delivery, gene expression, immune manipulation, and the development of molecular targets and stressed that all of these fields will need further advancement to make gene therapy a reality" (page 471, col. 1, last sentence of first paragraph). There are several factors known to limit the effectiveness of gene therapy, among which are the lack of optimal vectors, the lack of a long-term and stable transgene expression *in vivo*. With respect to the breadth of the instant claims encompassing the expression of any and all functional active proteins, factors such as the level of mRNA produced, the stability of the protein or polypeptide produced, the protein's proper compartmentalization within the cell or its secretory fate differ dramatically based on which protein or polypeptide being produced, and therefore the desired therapeutic effects sought to achieve. Thus, the level of transgene expression *in vivo*, its duration, and its *in vivo* therapeutic effects can not be predictable, nor these obstacles can be overcome with routine experimentation. With the lack of guidance provided by the instant disclosure regarding the above issues and in light of the unpredictable nature of the gene therapy art, it would have required undue experimentation without a predictable expectation of success for one skilled in the art to make and use the claimed invention.

Finally, it is also unclear whether the transplanted genetically modified bone marrow cells or oval cells could be stably

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

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**Supplemental Box**

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

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engrafted and differentiated properly into the treated patient. It should be noted that genetically modified cells may express gene products associated with the recombinant vectors used to deliver the desired transgene, e.g. adenoviral functional proteins. These and some of the transgene products themselves would also be recognized by the host immune system as foreign proteins and adverse immune reactions would be mounted against the transplanted genetically modified cells. As an example, Riddell et al. (Nature Med. 2:216-223, 1996) reported that five out of six patients seropositive for human immunodeficiency virus developed cytotoxic T-lymphocytes responses specific to a novel protein and eliminated infused autologous CD8+ HIV-specific cytotoxic T cells transduced with a suicide gene encoding a hygromycin phosphotransferase and the herpes virus thymidine kinase fusion protein. The instant disclosure fails to provide any guidance demonstrating that genetically modified bone marrow cells or oval cells would be able to avoid adverse-host immune rejection reactions upon transplantation into a patient in need of, such that they could be stably engrafted, proliferate, differentiate and express effective levels of desired transgene products to yield any therapeutic effects contemplated by the instant invention. In the absence of such guidance, it would again require undue experimentation without a predictable expectation of success for a skilled artisan to make and use the claimed invention.

Claims 1-19 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

**Response to Arguments**

Applicants argued that the instant application discloses methods for obtaining bone marrow, for enriching stem cells and hepatic oval cells, for administering stem cells or oval cells to a subject in need of transplantation. Applicants also argued that techniques for bone marrow transplantation are well known in the arts. Applicants further argued that Applicants have demonstrated in a rat animal model that transplanted bone-marrow derived cells and oval cells can participate in the production of hepatocytes and bile ductal cells.

Applicants' arguments are found to be unpersuasive because Applicants fail to address the specific issues set forth above. Nor do Applicants provide any factual evidence indicating one skilled in the art could have practiced the instant invention in its full scope without undue experimentation. Bone marrow transplantation is well known in the art, but the methods as claimed are not routine, particularly for achieving therapeutic effects contemplated by Applicants.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
31 August 2000 (31.08.2000)

PCT

(10) International Publication Number  
**WO 00/50048 A3**

(51) International Patent Classification<sup>7</sup>: A61K 35/28,  
35/407, A61P 1/16, 1/18 // C12N 5/06, 5/08

(74) Agents: SEIDE, Rochelle, K. et al.; Baker Botts LLP, 30  
Rockefeller Plaza, New York, NY 10112-0228 (US).

(21) International Application Number: PCT/US00/04744

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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(30) Priority Data:  
60/122,250 26 February 1999 (26.02.1999) US

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(71) Applicant (*for all designated States except US*): UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION [US/US]; 200 Gardner Steel Conference Center, Pittsburgh, PA 15260 (US).

Published:  
— With international search report.

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): PETERSEN, Bryon, E. [US/US]; 243 Willow Drive, Monroeville, PA 15146 (US). GOFF, Julie, P. [US/US]; 7531 Tuscarora Street, Pittsburgh, PA 15208 (US).

(88) Date of publication of the international search report:  
1 February 2001

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: BONE MARROW TRANSPLANTATION FOR HEPATIC REGENERATION AND REPAIR

(57) Abstract: The present invention relates to methods and compositions for stimulating liver regeneration in subjects with liver disorders. Specifically, the methods and compositions of the invention provide for the transplantation of bone marrow cells into a recipient host in amounts sufficient to result in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration. The invention is based in the observation that bone-marrow derived cells, can participate in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration. The present invention further provides methods for deriving enriched populations of hepatic oval cells, considered to be hepatic stem cells, utilizing antibodies that recognizes the Thy-1 cell surface antigen expressed on the surface of hepatic oval cells. The enriched populations of hepatic oval cells can be transplanted into a host for stimulating liver regeneration in subjects with liver disorders.

WO 00/50048 A3

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(Sheet 1 of 18)

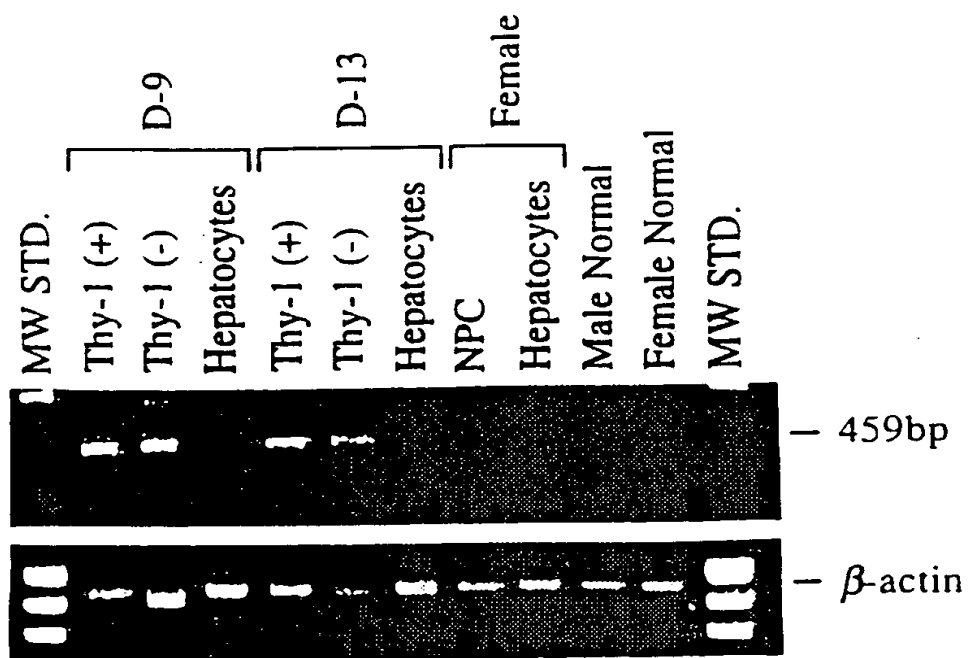


FIGURE 1

(continued)  
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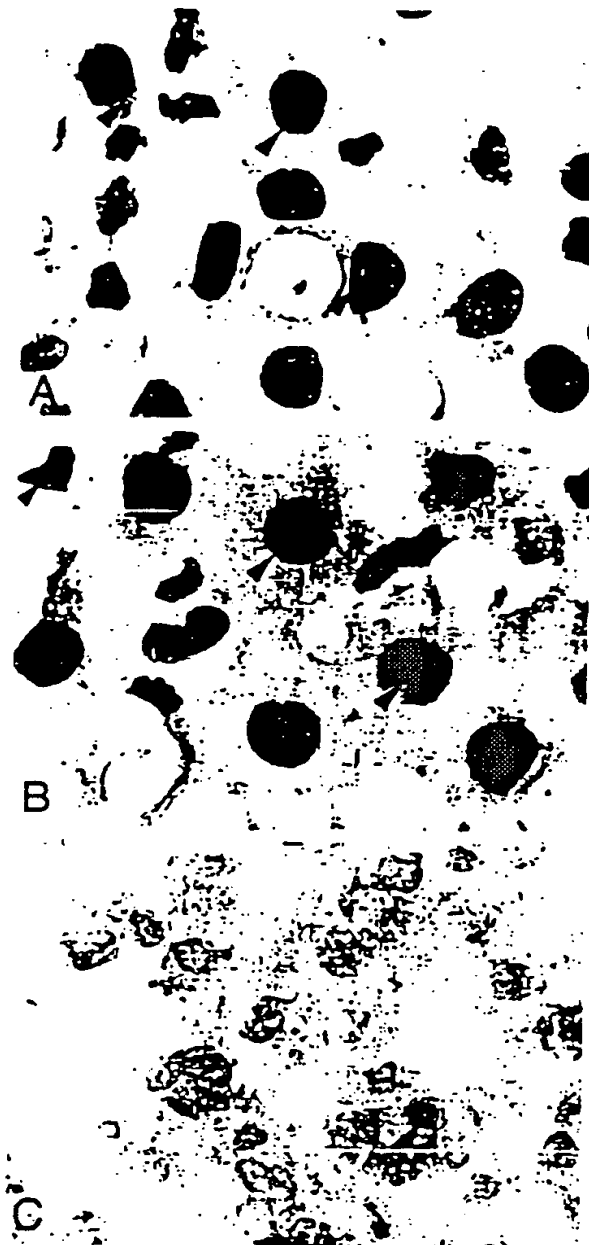
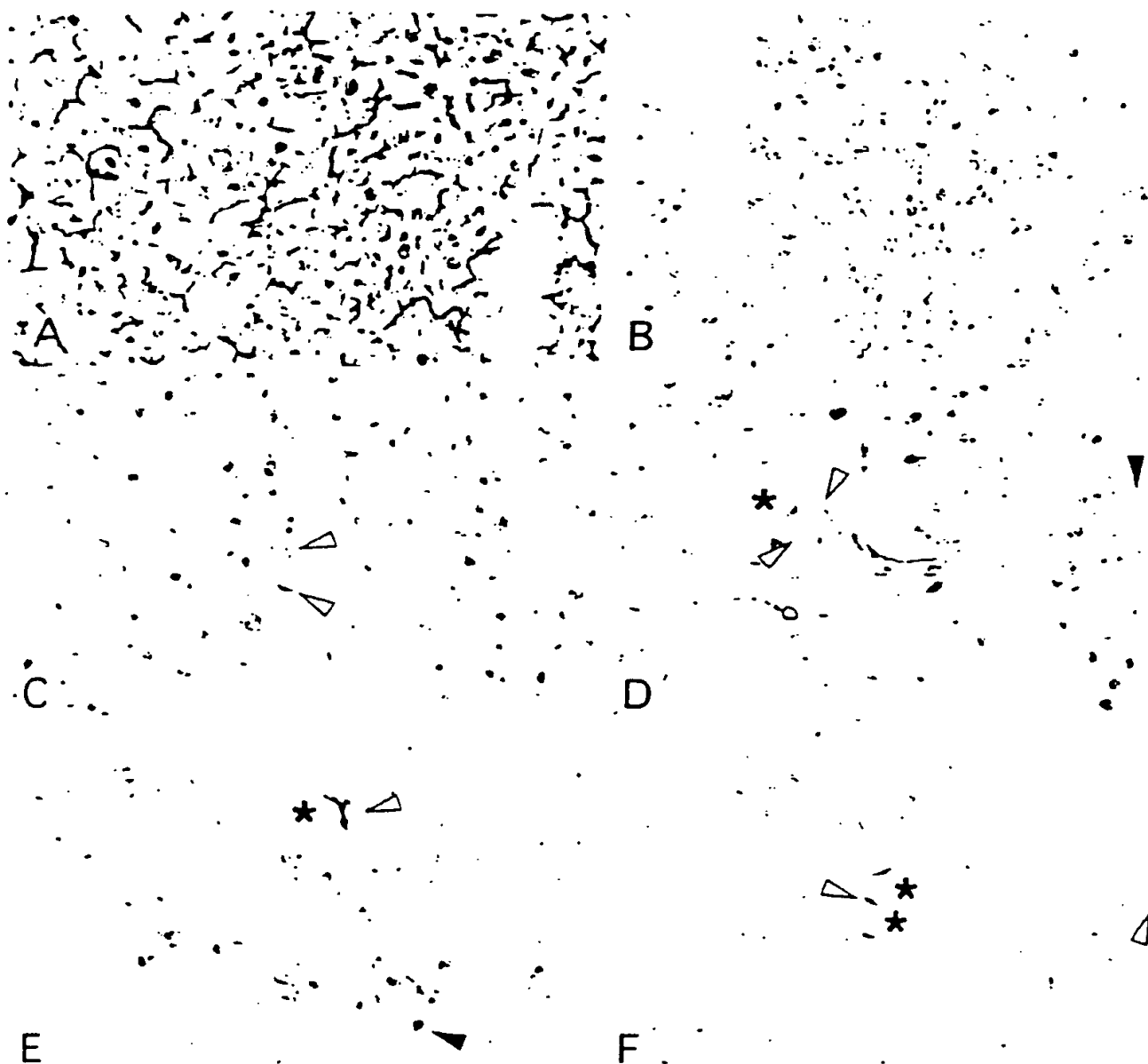
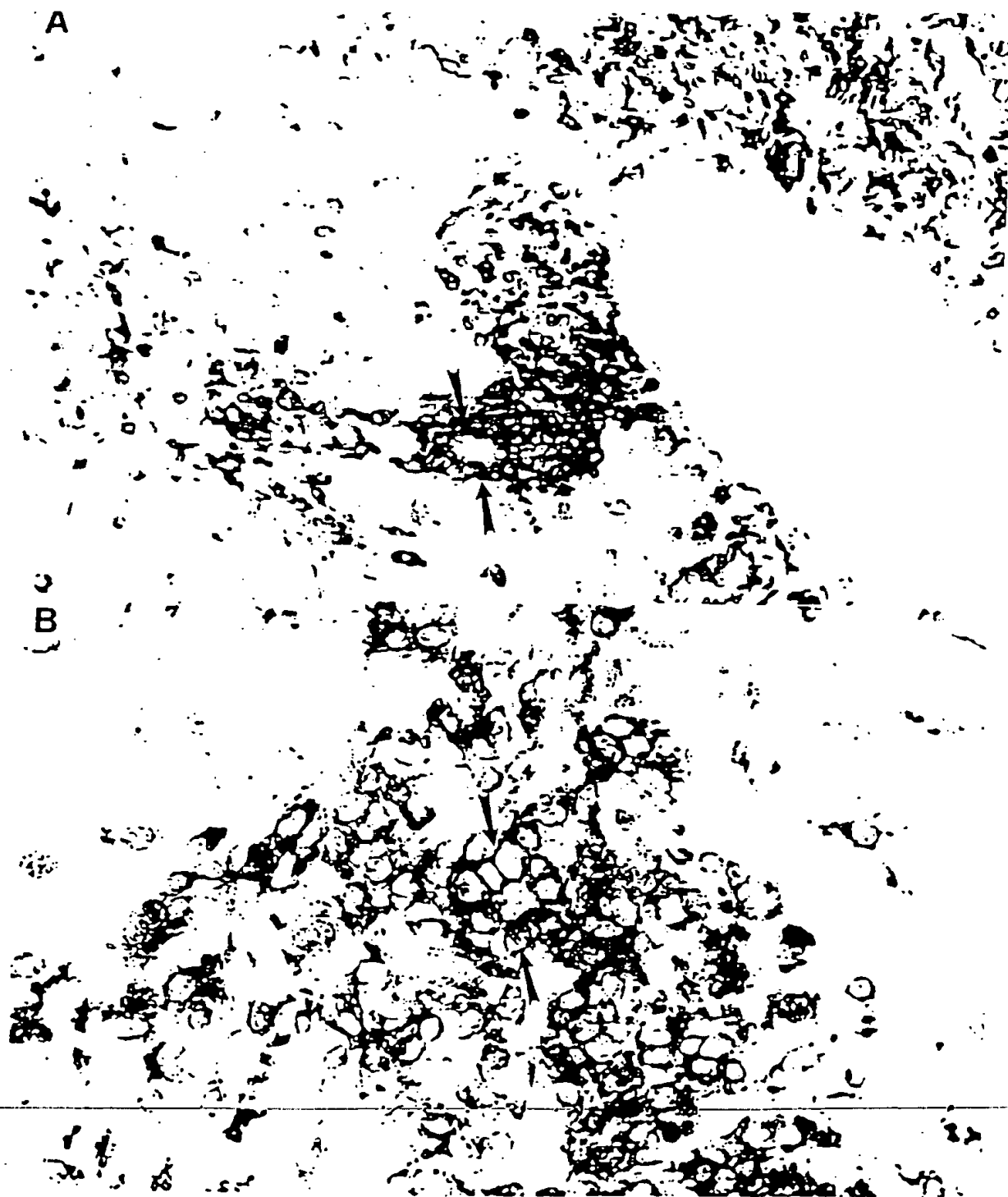


FIGURE 2

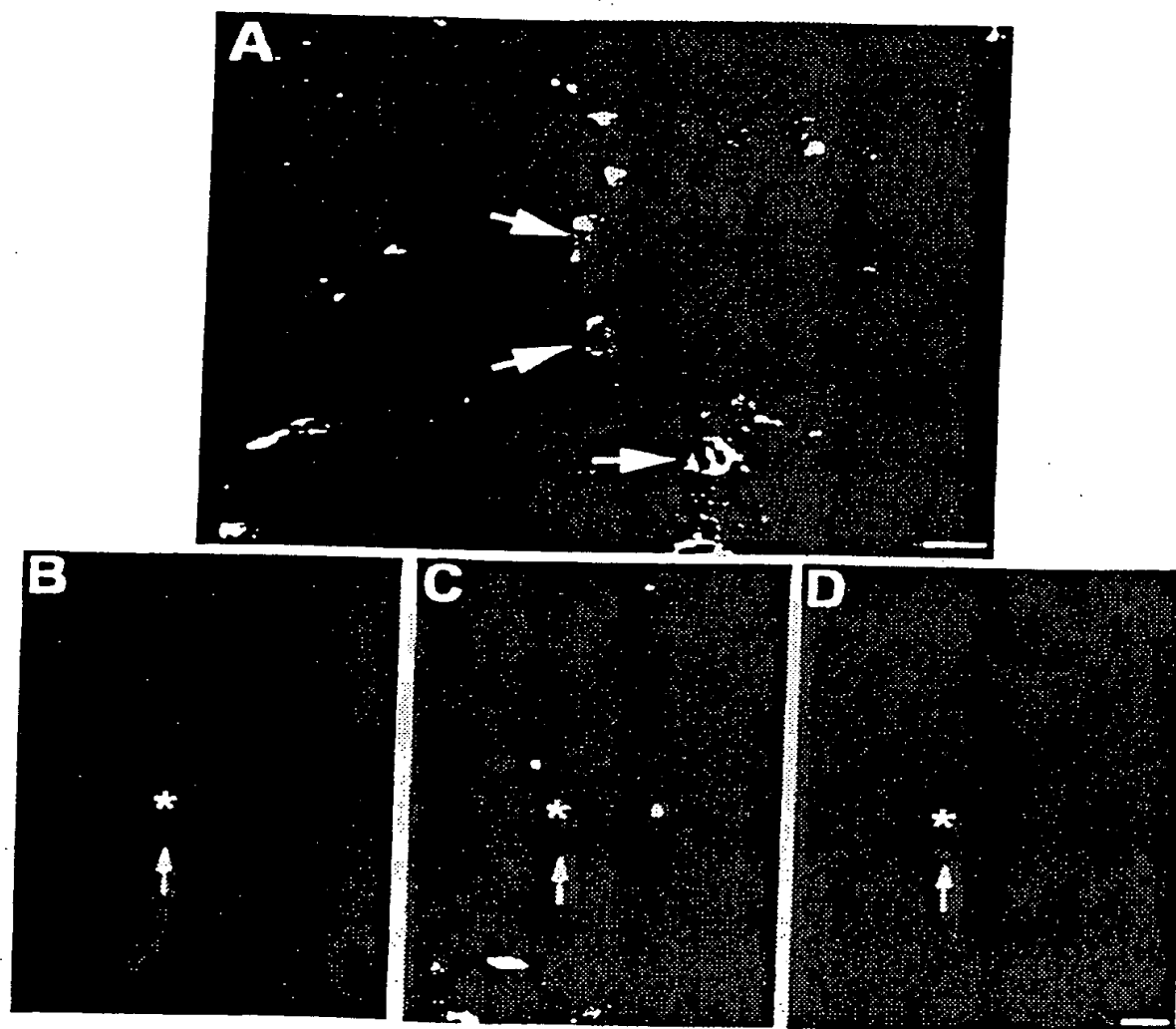
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FIGURE 3A-F





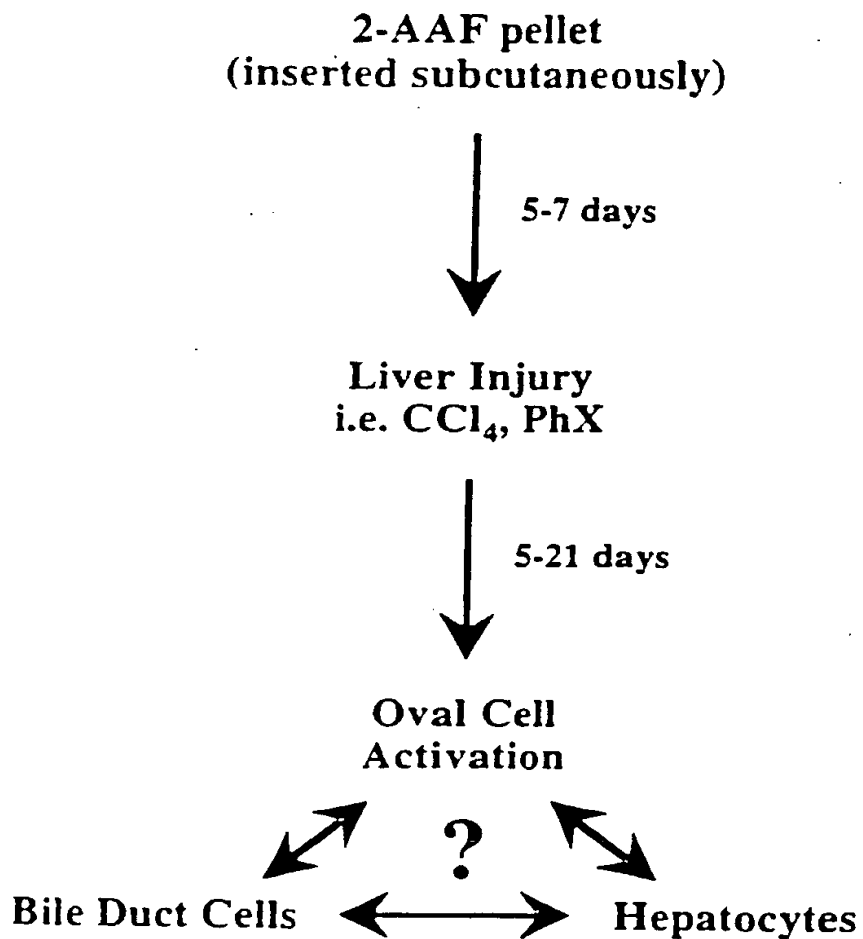
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FIGURE 5 A-D





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*FIGURE 6*

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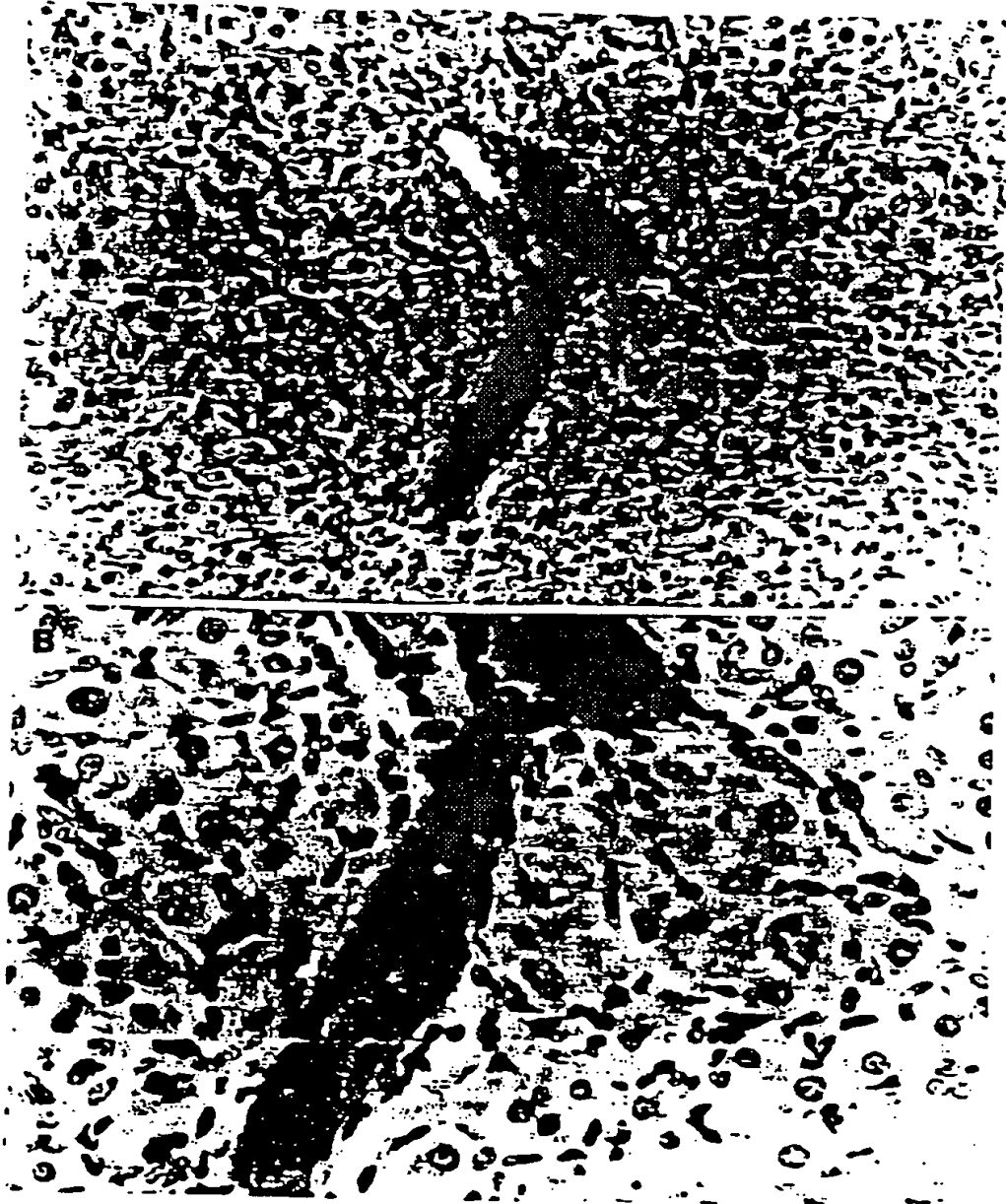


Figure 7A-B

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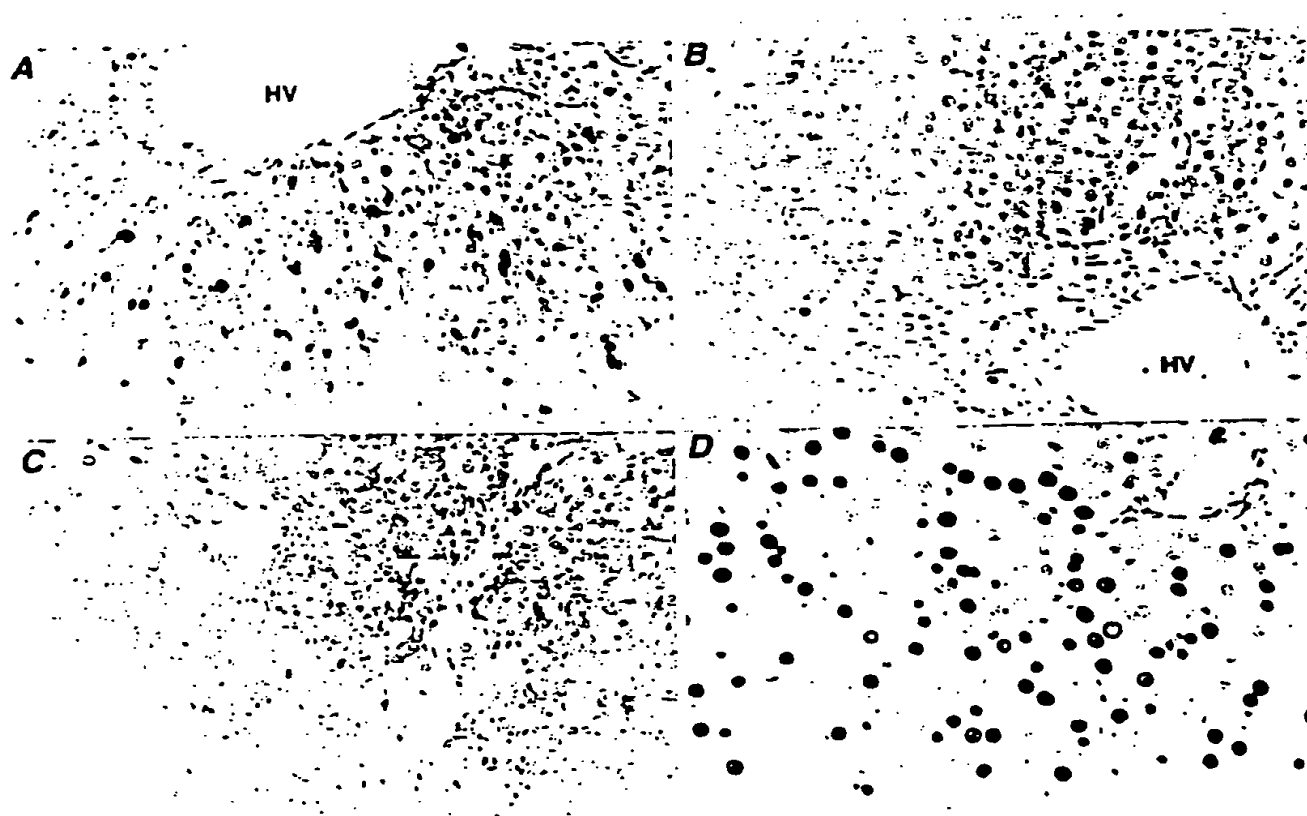


Figure 8A-D

(Sheet 9 of 18)

Figure 9A-B



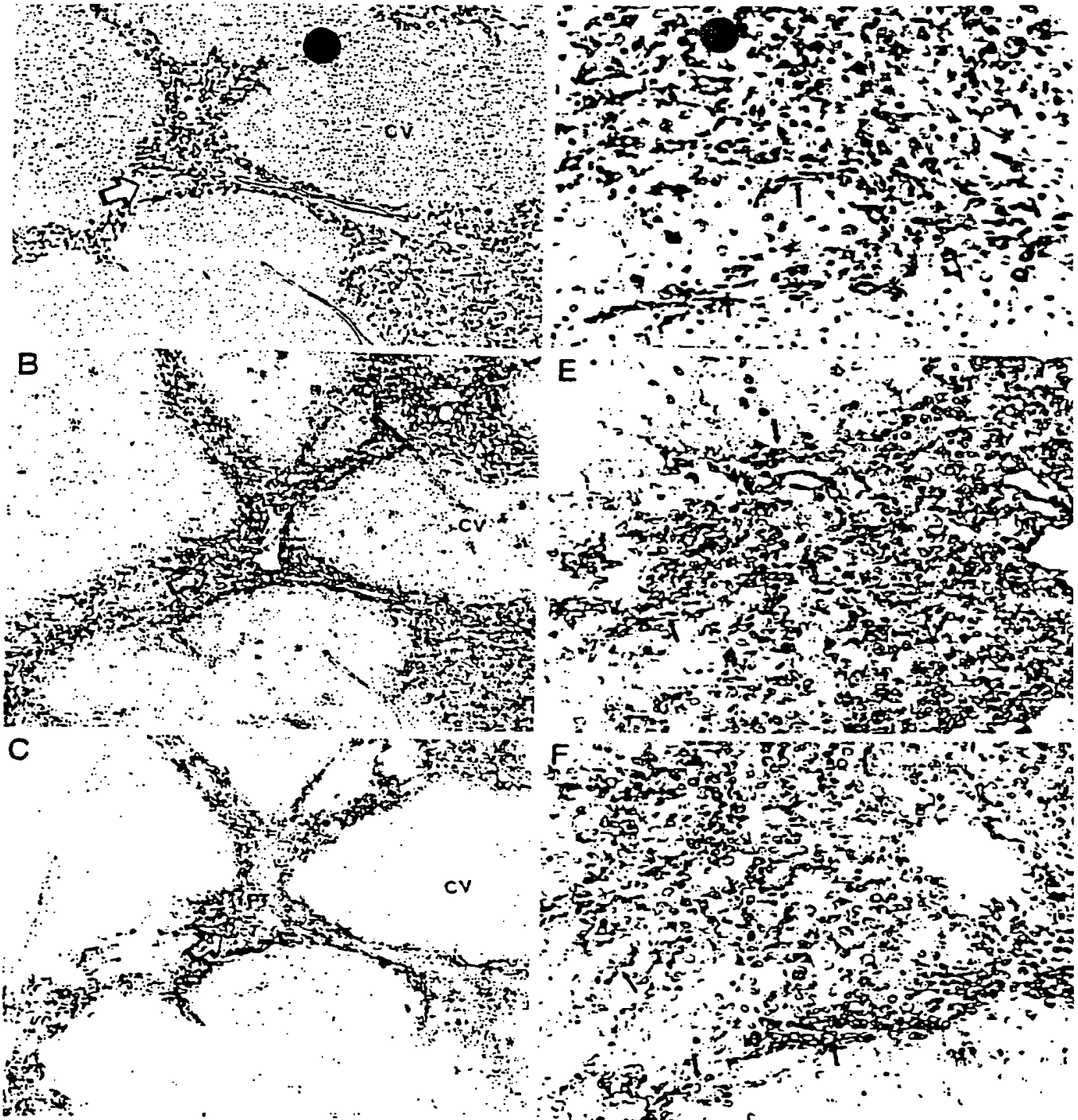


Figure 10A-F

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(Sheet 10 of 18)

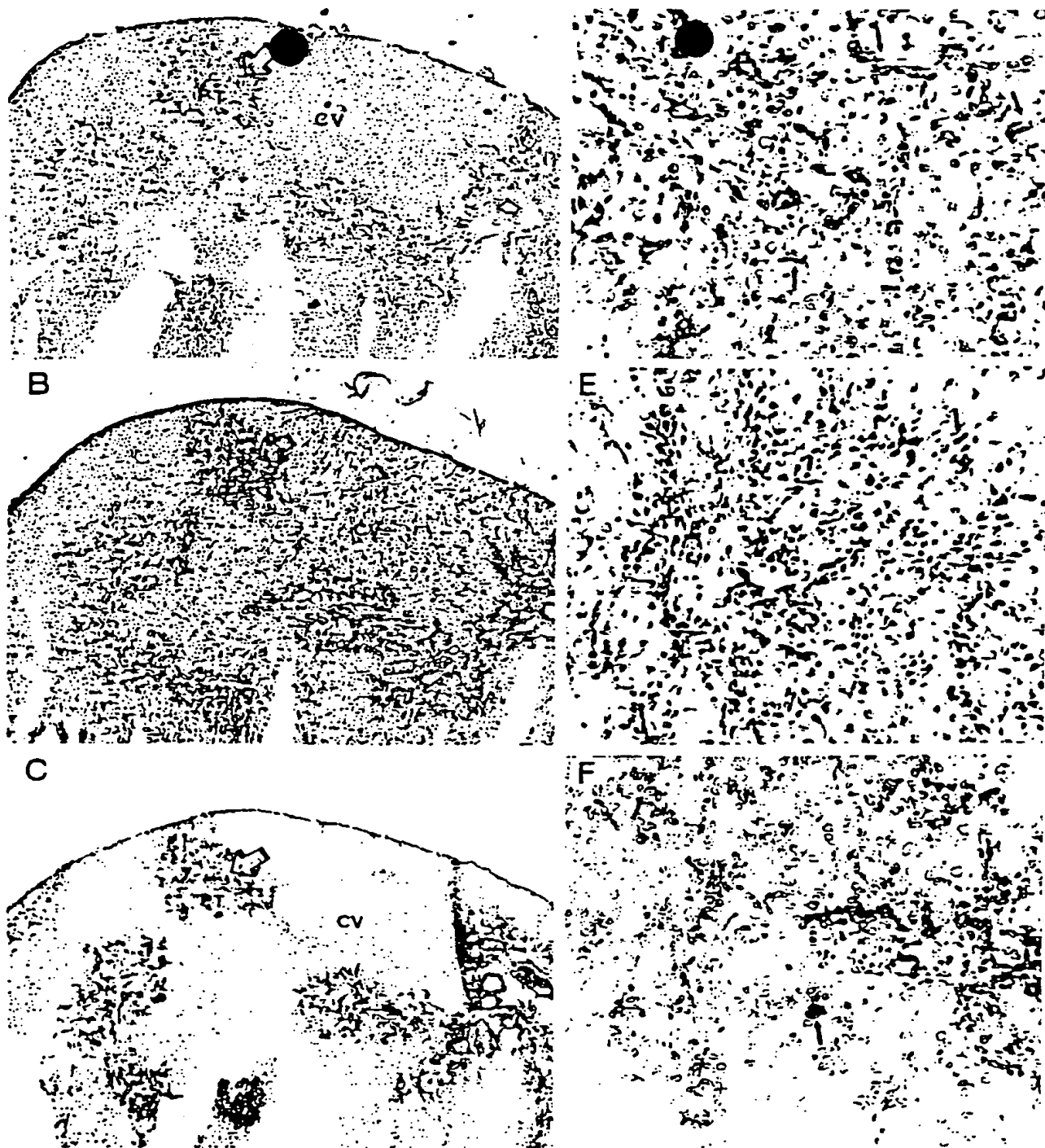


Figure 11 A-F

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(Sheet 12 of 18)

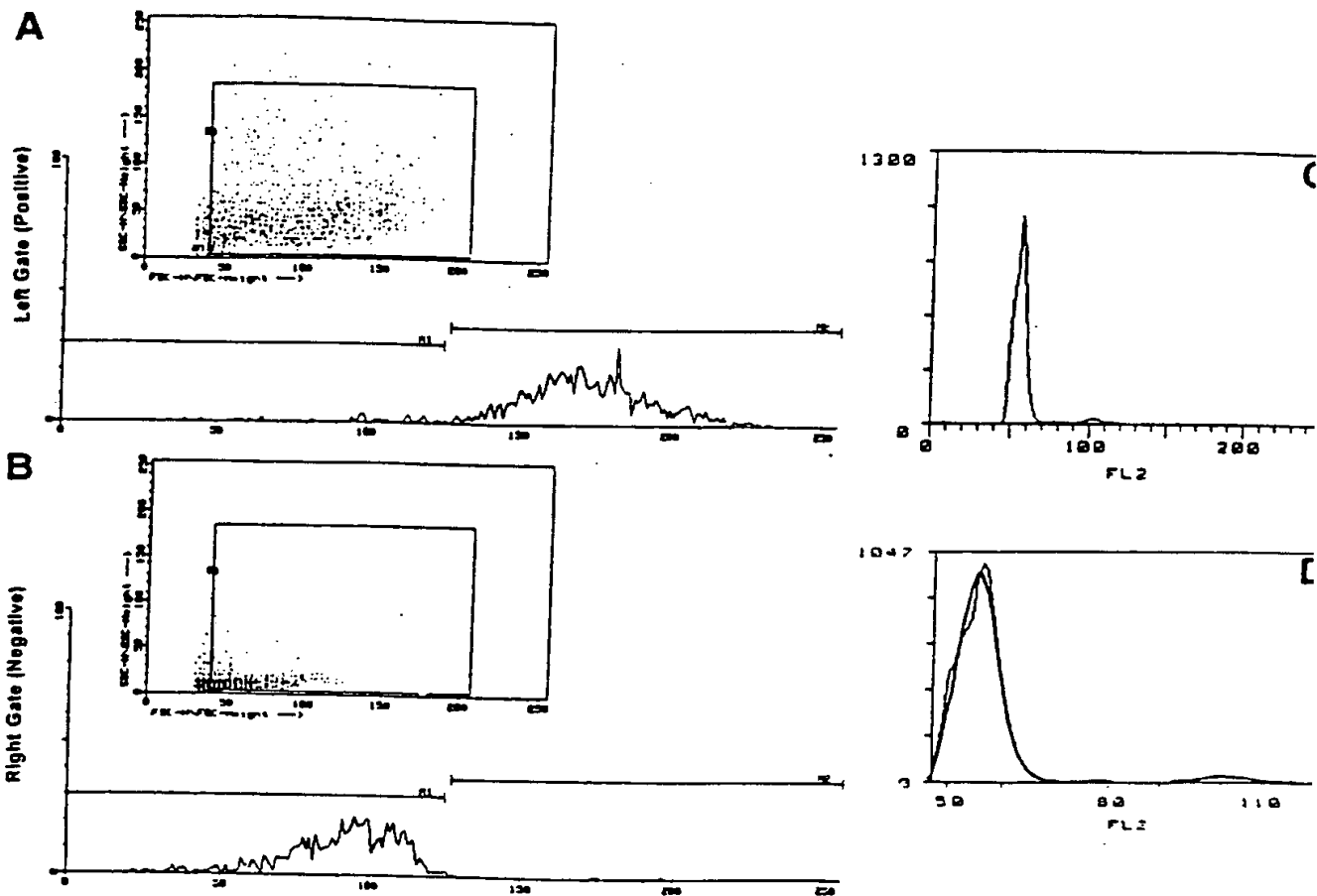


Figure 12 A-D

(Sheet 13 of 18)

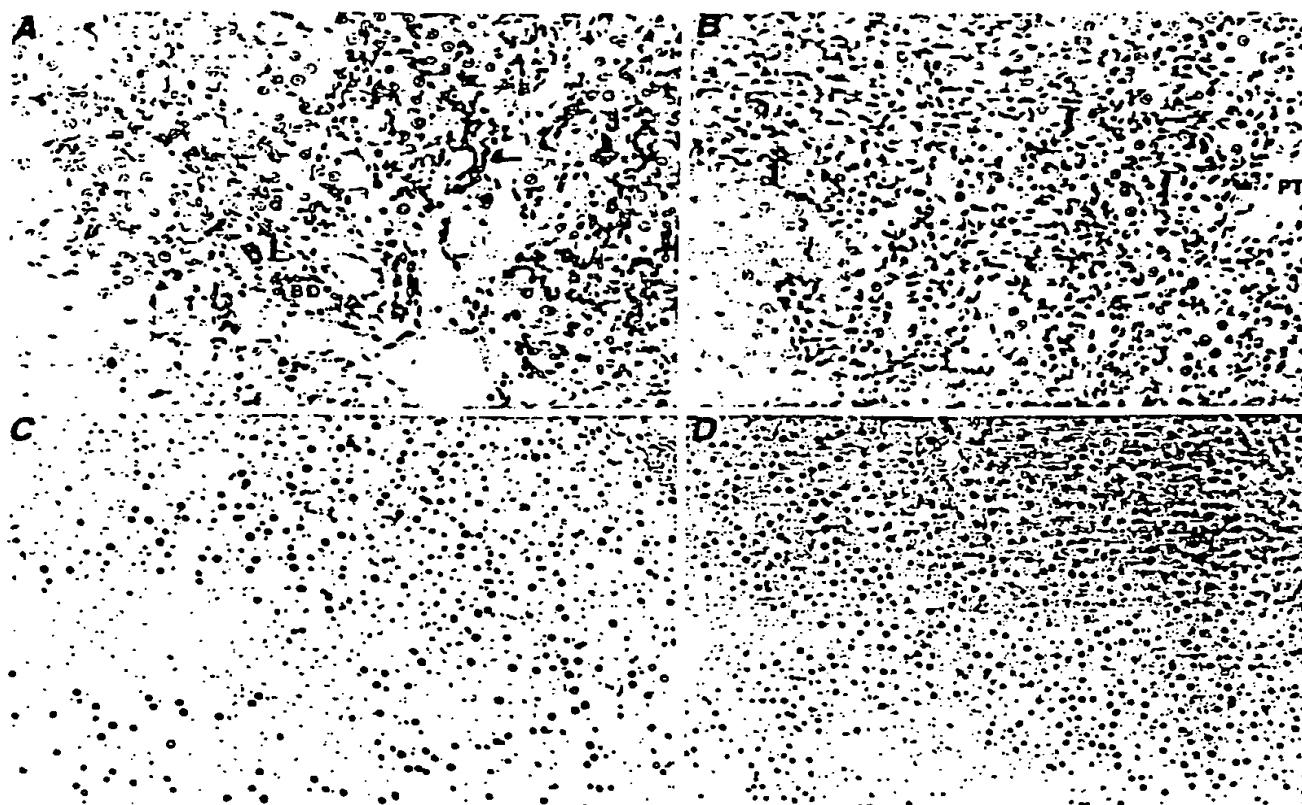


Figure 13A-D



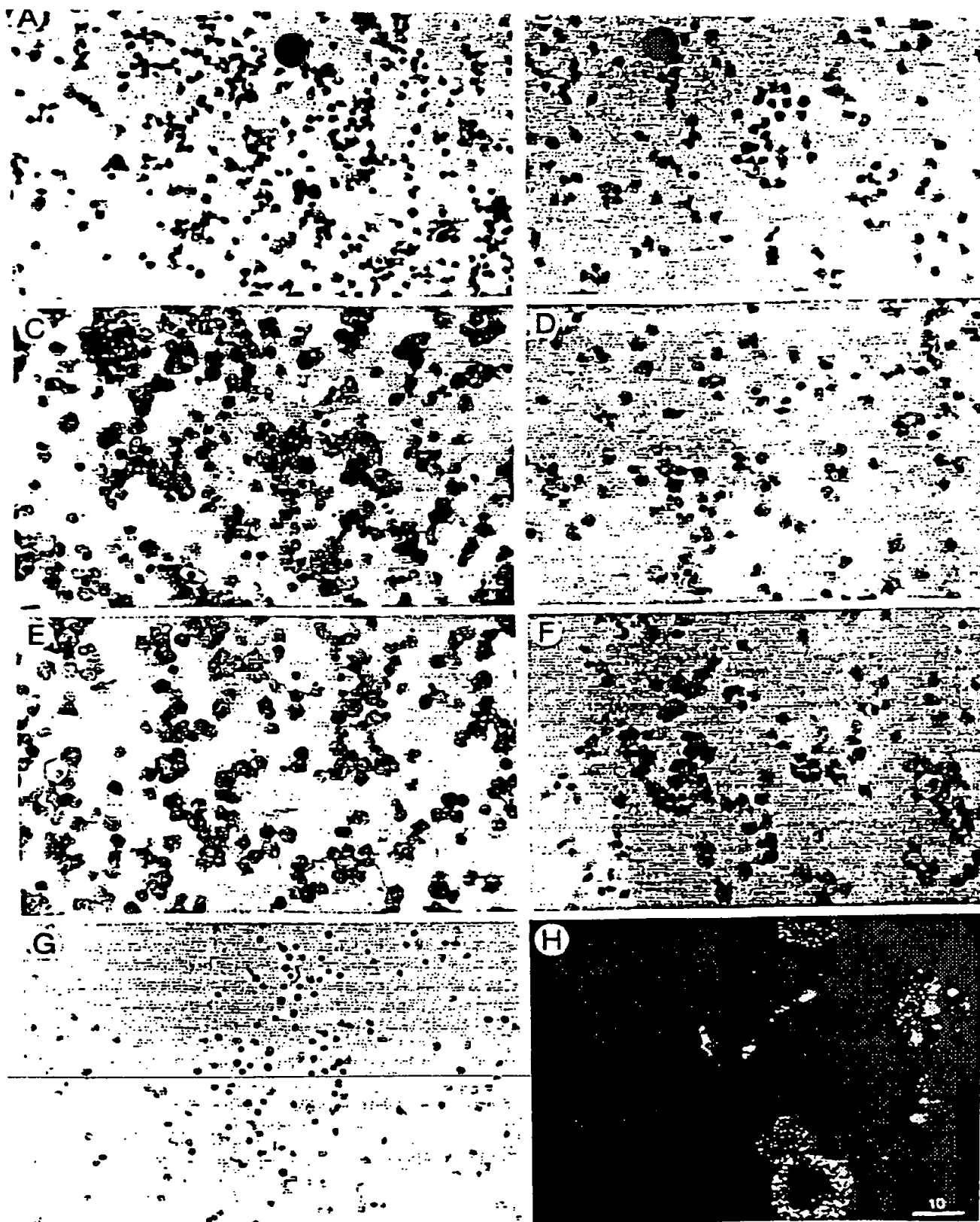
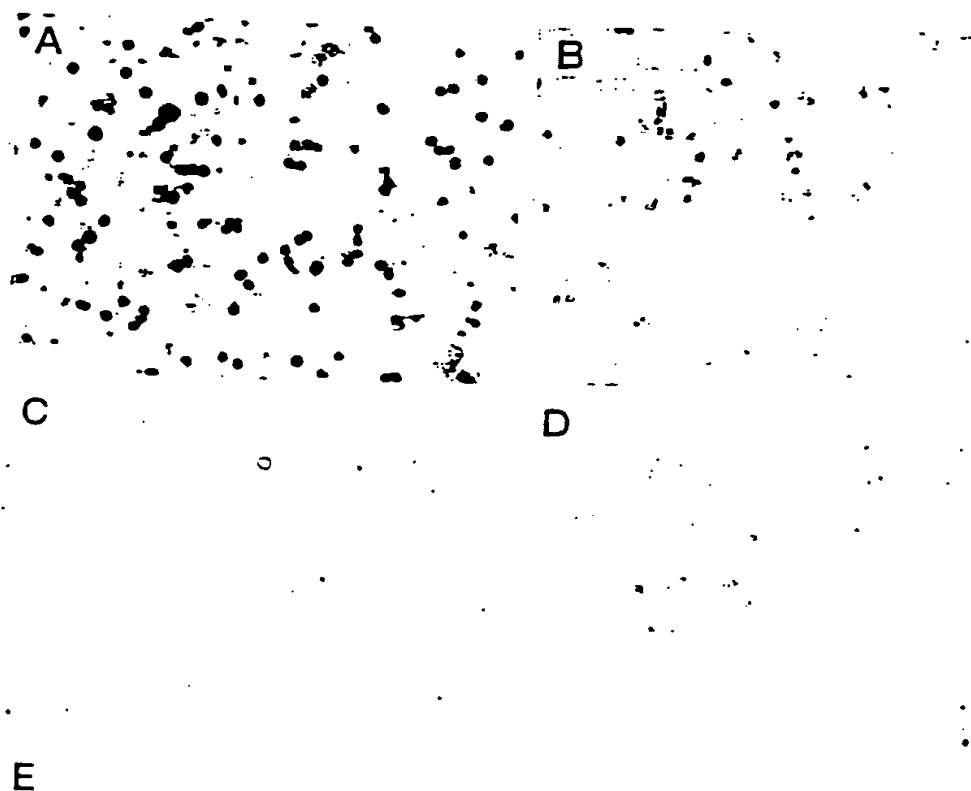
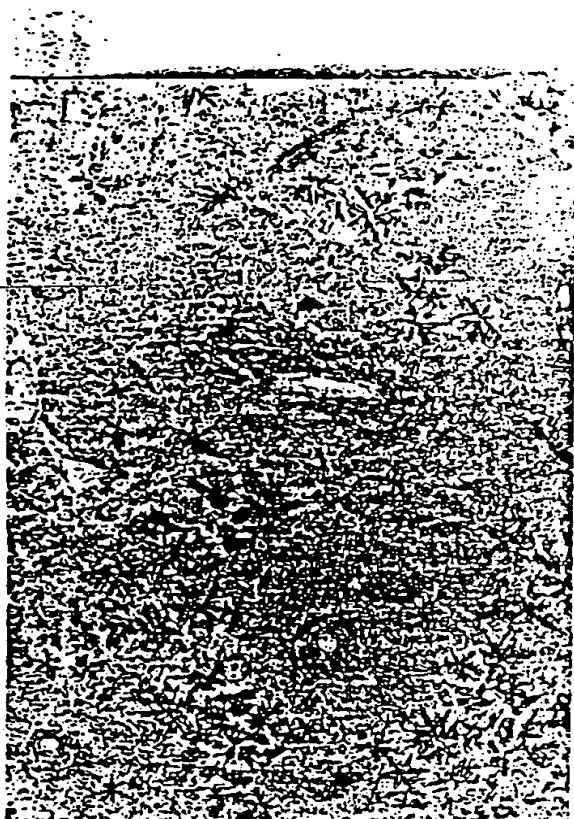


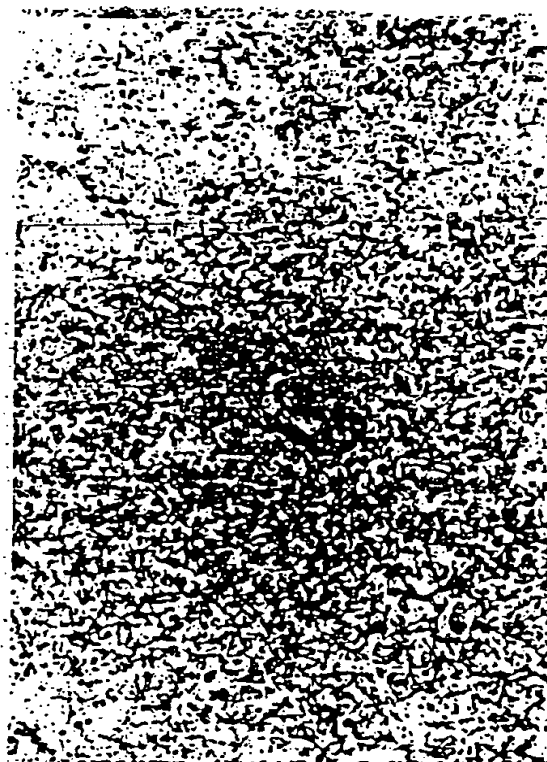
Figure 14A-H  
(Not 14 of 18)

A32212

*Figure 15A-E*



C



D

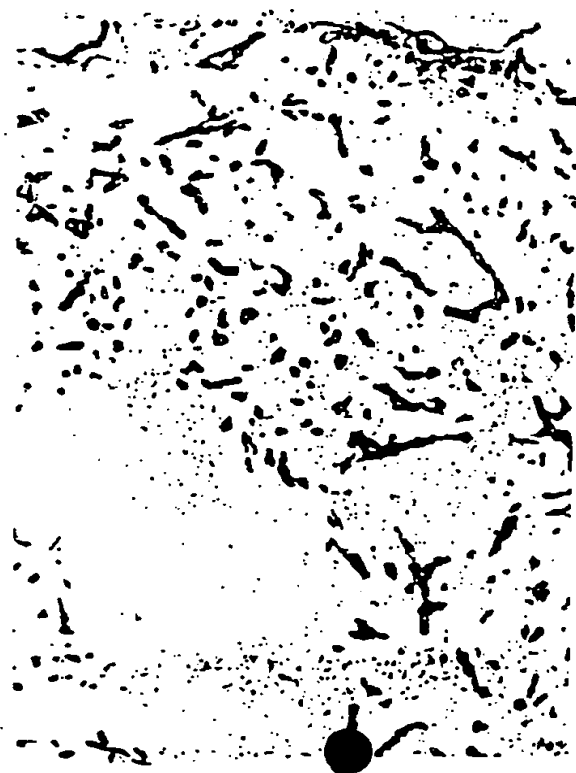
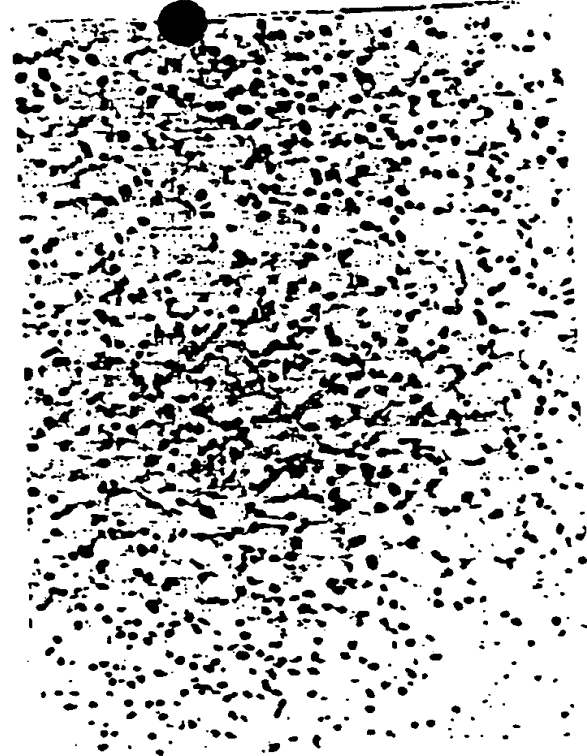


FIGURE 1A-D



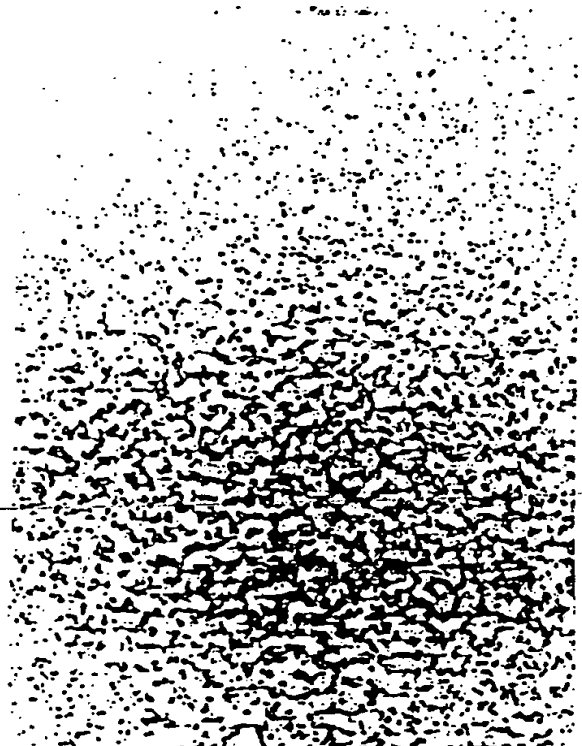
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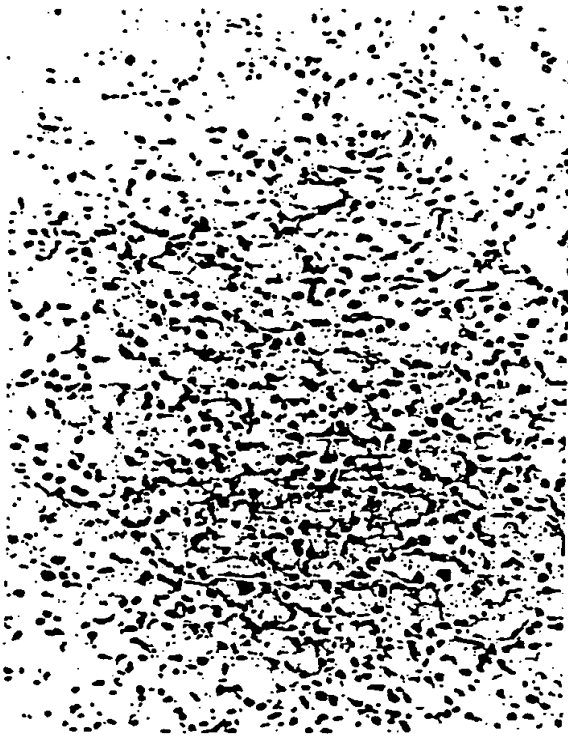


D.



A.





E.



F.

# INTERNATIONAL SEARCH REPORT

International Application No.  
PC./US 00/04744

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K35/28 A61K35/407 A61P1/16 A61P1/18 //C12N5/06,  
C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE, CANCERLIT, CHEM ABS Data, EMBASE, SCISEARCH, EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 789 246 A (REID LOLA M ET AL) 4 August 1998 (1998-08-04) the whole document	1-5, 11-13
X	PETERSEN BRYON E ET AL: "Thy-1+ sorted hepatic oval cells in culture maintain oval cell phenotypic markers." FASEB JOURNAL, vol. 12, no. 4, 17 March 1998 (1998-03-17), page A468 XP002147734 ISSN: 0892-6638 the whole document	20-23

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

18 September 2000

Date of mailing of the international search report

29/09/2000

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NL - 2280 HV Rijswijk  
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Authorized officer

Stein, A

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No

PCT/US 00/04744

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 5789246	A	04-08-1998	US 5576207 A	19-11-1996
			AU 667680 B	04-04-1996
			AU 2402092 A	02-03-1993
			CA 2115140 A	18-02-1993
			EP 0597964 A	25-05-1994
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			WO 9303142 A	18-02-1993
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WO 9939743	A	12-08-1999	AU 2590399 A	23-08-1999

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>32212-PCT</b>	<b>FOR FURTHER ACTION</b>		see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. <b>PCT/US 00/ 04744</b>	International filing date (day/month/year) <b>25/02/2000</b>	(Earliest) Priority Date (day/month/year) <b>26/02/1999</b>	
Applicant <b>UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH ....</b>			

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

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(54) Title: BONE MARROW TRANSPLANTATION FOR HEPATIC REGENERATION AND REPAIR

(57) Abstract: The present invention relates to methods and compositions for stimulating liver regeneration in subjects with liver disorders. Specifically, the methods and compositions of the invention provide for the transplantation of bone marrow cells into a recipient host in amounts sufficient to result in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration. The invention is based in the observation that bone-marrow derived cells can participate in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration. The present invention further provides methods for deriving enriched populations of hepatic oval cells, considered to be hepatic stem cells, utilizing antibodies that recognizes the Thy-1 cell surface antigen expressed on the surface of hepatic oval cells. The enriched populations of hepatic oval cells can be transplanted into a host for stimulating liver regeneration in subjects with liver disorders.

## **BONE MARROW TRANSPLANTATION FOR HEPATIC REGENERATION AND REPAIR**

### **SPECIFICATION**

#### **1. INTRODUCTION**

5           The present invention relates to methods and compositions for stimulating liver regeneration in subjects with liver disorders. Specifically, the methods and compositions of the invention provide for the transplantation of bone marrow cells into a recipient host in amounts sufficient to result in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration. The invention is  
10       based in the observation that bone-marrow derived cells, can participate in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration.

          The present invention further provides methods for deriving enriched populations of hepatic oval cells, considered to be hepatic stem cells, utilizing antibodies that recognizes the Thy-1 cell surface antigen expressed on the surface of  
15       hepatic oval cells. The enriched populations of hepatic oval cells can be transplanted into a host for stimulating liver regeneration in subjects with liver disorders. The present invention, by enabling methods for the transplantation of bone marrow cells and/or oval cells for stimulation of liver regeneration provides a safer alternative to whole liver transplantation in subjects having liver disorders including, but not limited  
20       to, cirrhosis of the liver, alcohol induced hepatitis, chronic hepatitis, primary sclerosing cholangitis and alpha<sub>1</sub>-antitrypsin deficiency.

#### **2. BACKGROUND OF INVENTION**

          The origin of the hepatic oval cell has been a topic of considerable interest and controversy for the past several decades. Because oval cells proliferate  
25       when hepatocytes are prevented from proliferating in response to liver damage, these cells have been considered to be hepatic stem cells, or the intermediate progeny of a hepatic stem cell. The prevailing opinion is that oval cells originate either from cells

present in the canals of Herring ( Grisham, J.W. and Thorgiersson, S.S. in Stem Cells, C.S. Potter, Ed. (Academic Press, San Diego, CA 1997) pp. 233-282) or from blast-like cells located next to bile ducts (Novikoff, P.M. et al., 1996, Am J. Pathol. 148:1473 ). Oval cells are not easily detected in normal livers. In certain pathological conditions, however, in which an inhibition of hepatocyte proliferation is followed by severe hepatic injury, oval cells are readily apparent due to their active proliferation. In recognized experimental animal models, hepatocyte proliferation is generally suppressed by exposure of the animal to 2-acetylaminofluorene (2-AAF) and, subsequently, hepatic injury is usually induced by partial hepatectomy (PHx) or by administration of carbon tetrachloride (CCl<sub>4</sub>) (Evarts, R.P., et al., 1989, Cancer Res. 49:1541; Petersen, B.E. et al., 1998, Hepatology 27:1030). Oval cells have been observed in organs other than the liver, such as the pancreas of rats fed a copper-deficient diet (Bartles, J.R. et al., 1991, J Cell Science 98:45; Rao, M.S. and Reddy, J.K., 1995, Seminars in Cell Biology 6:151).

Throughout life, hepatic and hematopoietic cells intermingle and appear to be interdependent. During fetal life, hematopoietic stem cells (HSC) move out of the yolk sac and take up residency in the liver, and until the time of birth, the liver functions as a hematopoietic organ (Baker, J.E. et al., 1969, J Cell Physiol 74:51; Moore, M.S. et al., 1970, Br J Haematol 18:279). This function ceases in the neonate, but under certain conditions it can be reactivated in the form of extra-medullary hematopoiesis (Tsamanda, A.C., 1995, Modern Pathol 8:671). The adult liver has been shown to harbor a significant number of HSC (Hayes, E.F. et al., 1975, J Cell Physiol 86:213), and it has been shown that the bone marrow of lethally irradiated animals can be reconstituted by whole liver transplantation (Murase N et al., 1996 Transplantation 61:1).

Hematopoietic activity and erythropoietic cells have been shown to reappear in the liver during liver regeneration following a partial hepatectomy (Naughton, B.A. et al., 1982, Exp Hematol 10:451; Barbera-Guillem, E. et al., 1989 Hepatology 9:29; Sakamoto, T., et al, 1992, Reg Immunol 4:1). It has also been shown that cultured rat hepatocytes can produce granulocyte-macrophage colony stimulating factor, a known hematopoietic cytokine (Sakamoto, T. et al., 1991, Reg

Immunol 3:260). Recently, oval cells were found to express CD34, Thy-1 and c-kit mRNAs and proteins (Petersen, B. et al., 1998, Hepatology 27:433; Omori, N. et al, 1997, Hepatology 26:720; Fujio, K. et al., 1994, Lab Invest 70:511). These antigens are known to be expressed on HSC. In addition, oval cells express *flt-3* receptor  
5 mRNA, which in humans and mice has been reported to be restricted to populations of hematopoietic progenitor cells (Omori, M. et al., 1997, Am J Pathol 150:1179). The adult mammalian liver can therefore be considered a potential hematopoietic organ.

### 3. SUMMARY OF THE INVENTION

The present invention provides methods and compositions for  
10 stimulating liver regeneration in subjects with liver disorders. The compositions and methods of the invention provide for the transplantation of bone marrow cells into a recipient in amounts sufficient to result in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration. The invention is based on the observation that bone-marrow derived cells, can participate in the production of  
15 hepatocytes, bile ductal cells and oval cells during liver regeneration.

The present invention further provides methods for deriving enriched populations of hepatic oval cells, considered to be hepatic stem cells, utilizing antibodies that recognizes the Thy-1 cell surface antigen expressed on the surface of hepatic oval cells. The enriched populations of hepatic oval cells may also be utilized  
20 in methods directed to regeneration of liver tissue.

The present invention also provides compositions for use in stimulating liver regeneration comprising bone marrow cells and/or hepatic oval cells in a pharmaceutical acceptable carrier. The compositions of the invention may be utilized for treatment of subjects with liver disorders where the stimulation of liver  
25 regeneration is desired. Such disorders include cirrhosis of the liver, alcohol-induced hepatitis, chronic hepatitis, primary sclerosing cholangitis, alpha<sub>1</sub>-antitrypsin deficiency and liver cancer.

In an embodiment of the invention, the bone marrow derived stem cells and/or hepatic oval cells may be genetically engineered, prior to transplantation, to  
30 enable them to produce a wide range of proteins, including but not limited to, growth

factors, cytokines, or biologically active molecules, such as hormones. In this way, any new liver tissue derived from the transplanted stem cells or hepatic oval cells will produce the desired biologically active protein.

5 The invention further relates to the *in vitro* attachment of stem cells or hepatic oval cells to a matrix prior to transplantation for the purpose of increasing the viability and growth of the transplanted cells. In addition, the matrix may be composed of additional materials including other types of cells or biologically active molecules.

#### 4. BRIEF DESCRIPTION OF THE DRAWINGS

10 FIGURE 1. PCR analysis of DNA from female rats transplanted with bone marrow from the femurs of male donor rats. The data presented here show that the transplanted animals tested positive for the Y chromosome in both the Thy1.<sup>+</sup> and Thy1.<sup>-</sup> sub-populations of non-parenchymal cells (NPC) at both days tested. The Thy-  
1<sup>+</sup> fraction shows strong signal probably due to the presence of hematopoietic cells.  
15 After successful BMTx, presumably the mature (differentiated) male hematopoietic cells could be found in the NPC population. The mature hematopoietic (i.e. Kupffer cells, monocytes, etc.) cells will be negative for Thy-1, but positive for the Y chromosome. Note the Day 13 fraction of hepatocytes are now expressing the Y chromosome PCR product. This is the time point when oval cells begin to make the  
20 transition to hepatocytes. As noted in the text, the Day 9 hepatocyte fraction was negative for the Y chromosome. This would be expected in the prevailing view of the timing events in the cascade of oval cell proliferation and differentiation. The control female DNA as well as the non transplanted female DNA is negative for Y chromosome expression. The  $\beta$ -actin product reveals that the DNA was present and  
25 intact.

FIGURE 2 A-C. Photomicrographs of *in situ* hybridizations of the Y chromosome *sry* gene performed on frozen liver sections. The arrows indicate positive reaction in the nucleus of hepatocytes. A, untreated control normal male rat (positive control); B, female treated with BMTx and the 2-AFF/CCl<sub>4</sub> protocol and  
30 sacrificed at day 13 following hepatic injury; and C, untreated female (negative

control). It should be noted that the color reaction-time for the untreated female was 45 minute, whereas the color reaction-time for both the male and the BMTx/2-AAF/CCl<sub>4</sub> treated female was only 5 minutes. The magnification of all the photomicrographs is 1000X.

5                   FIGURE 3 A-F. DPPIV activity in frozen liver sections. A, untreated DPPIV<sup>+</sup> rat used as a positive control. A diffuse decoration of the bile ductular site of hepatocytes is evident (orange color). B, untreated DPPIV<sup>-</sup> rat showing a complete absence of DPPIV activity (negative control). C-F are four different BMTx (male DPPIV<sup>+</sup> donor) DPPIV<sup>-</sup> rat (recipient) exposed to the 2-AAF/CCl<sub>4</sub> protocol for oval cell induction and sacrificed at day 11 or day 13 following hepatic injury. A positive reaction is evident not only between hepatocytes from all four animals, but also a on a few oval or transitional cells (D and E). The DPPIV staining can appear as a line (open arrowheads) or as a dot (closed arrowheads) depending the plane of the section through the bile cannilcuar region. In all cases there are clusters of cells (2-5)  
10                   expressing the DPPIV marker. Hepatocytes that are from donor origins are denoted by \*. Original magnifications, 200x.  
15

                  FIGURE 4 A-B. Sections from two Brown-Norway rat livers, transplanted into Lewis recipient rats, sacrificed 11 days (A) and 13 days (B) after CCl<sub>4</sub> administration in the oval cell induction protocol. The sections were  
20                   immunostained with an L21-6 mAb. Positive oval cells (arrow heads) and positive cells in ductal structures (arrows) can be seen. Original magnifications, A, 100x, and B, 200x.

                  FIGURE 5 A-D. Frozen liver section showing double immunofluorscence staining of a periportal region in a Brown-Norway liver  
25                   transplanted into a Lewis recipient rat (A). Green fluorescence: anti L21-6, a recipient marker; red fluorescence: anti OC-2, an oval cell and a mature ductal cell marker. When the two antibodies are in close proximity, the light waves mix and the emitted fluorescence is yellow. Oval cells co-expressing the two markers are evident (arrows). Other cells can be seen that express only L21-6 (presumably inflammatory  
30                   cells), or only OC.2 (oval cells that could have been derived from, L21-6 negative precursor-cells resident in the Brown-Norway liver). B-D shows a frozen section of a



Brown-Norway liver transplanted into a Lewis recipient rat and put on the 2-AAF/ $\text{CCl}_4$  protocol, day 13 after  $\text{CCl}_4$  exposure. Centered in the photomicrograph is a ductal structure (B-D), the same type of structure seen in Figure 4A. Double immunofluorescence staining was performed using anti-L21-6 (B) and anti-OC-2 (D).  
5 Merged images of the L21-6 and OC-2 immunofluorescence staining are shown in the middle photomicrograph (C). Cells expressing both antigens are yellow (C). As a point of origin, \* indicates the center of the bile duct. The origin of the cells within this duct is extrahepatic because they are positive for L21-6, which makes them recipient derived cells. Arrows in B-D indicate the same cell in all three  
10 photomicrographs. Bars represent 10  $\mu\text{m}$  in length.

FIGURE 6. Time line of events for activation of oval cell proliferation. The presence of 2-AAF is necessary to suppress hepatocyte proliferation and to allow extended proliferation of oval cells. The diagram represents the different stages of oval cell proliferation.

15 FIGURE 7 A-B. Liver section obtained from a rat on day 11 on the 2-AAF/ $\text{CCl}_4$  protocol. Sections were stained with hematoxylin-eosin. Centered in the photomicrograph is a portal triad (PT). The small oval cells (*arrows*) can be seen between the larger hepatocytes. (Original magnification [A] X100; [B] X200.)

FIGURE 8 A-D. Immunohistochemistry for BrdU incorporation on  
20 liver sections obtained from animals on the 2-AAF/ $\text{CCl}_4$  protocol at various time points post-hepatic injury. (A) Day 9; (B) day 11; (C) day 13; and (D) positive control for BrdU at the 24-hour Phx time point. The peak of proliferation occurs at day 9, with a drastic drop-off of labeling on subsequent days. The day 13 time point is also represented in Fig. 8. HV, hepatic vein. (Original magnification [A-D] X200.)

25 FIGURE 9 A-B. Frozen liver sections obtained from normal rat liver. Histological appearance of liver section stained with (A) Thy1.1 antibody and (B) OC.2 antibody. Normal rat liver was used as a negative control for immunostaining procedures. Thy1.1 expression cannot be detected in a normal adult liver, and OC.2 can be detected only on the ductular epithelium in the portal triads.  
30 The portal triad region (PT) and central vein region (CV) are designated in the lobule. (Original magnification X40.)

FIGURE 10 A-F. Frozen serial sections from rat liver exposed to the 2-AAF/ $\text{CCl}_4$  protocol (day 21). (A and D) Immunohistochemical expression of CK-19. (B and E) expression of OC.2. (C and F) Expression of Thy1.1 staining as can be seen at lower magnification (A-C), CK-19, OC.2, and Thy1.1 exhibit nearly the same staining pattern. At higher magnification (D-F), the individual oval cells stain positive for each antibody. Open *arrows* in (A-C) indicate the reference point at which the photomicrographs of higher magnifications were taken. *Solid arrows* in (D-F) show individual oval cells positively stained for the appropriate antibody. It should be noted that the ductular epithelium is now staining positive for Thy-1. In normal liver, this is not the case. This same result can be seen in Fig. 6 as well. (Original magnification [A-C] X40; [D-F] X200.)

FIGURE 11 A-F. Frozen serial sections from rat liver exposed to 2-AAF/PHx protocol (day 13). (A and D) Immunohistochemical expression of CK-19. (B and E) Expression of OC.2. (C and F) Expression of Thy1.1 staining. At lower magnification (A-C), CK-19, OC.2, and Thy1.1 exhibit nearly the same staining pattern. At higher magnification (D-F), the individual oval cells stain positive for each antibody. *Open arrows* in (A-C) indicate the reference point at which the photomicrographs of higher magnifications were taken. *Solid arrows* in (D-F) show individual oval cells positively stained for the appropriate antibody. This figure illustrates that, in this model, there are no inflammatory cells to confound the issue as to which cell type is expressing Thy-1. Staining patterns are alike for all three markers, and this pattern is similar to what is seen in the 2-AAF/ $\text{CCl}_4$  model. (Original magnification [A-C] X40; [D-F] X200.)

FIGURE 12 A-D. Profiles of NPC fraction obtained from perfused rat liver exposed to the 2-AAF/ $\text{CCl}_4$  protocol. Cells were labeled with Thy1.1-FITC antibody and sorted by flow cytometry. Cells were separated into two factions: (A) right gate, Thy-1<sup>+</sup>-labeled cells and (B) left gate, Thy-1<sup>-</sup> cells. Histograms revealed that 95% to 97% purity could be obtained for Thy-1.1<sup>+</sup> cells, while 99% purity could be achieved for negative cells. (C) Sorted Thy1.1<sup>+</sup> cells were stained with PI, and flow cytometric cell cycle analysis was performed. Analysis revealed that 94% Thy1.1<sup>+</sup> cells are in the G0/G1 (resting) stage of the cell cycle. (D) Thy-1-labeled

cells (*dark solid line*) that are in the G0/G1 stage of the cell cycle (*dashed line*). Approximately 90% of the resting cells are Thy-1<sup>+</sup>.

FIGURE 13 A-B. Immunohistochemistry of liver section obtained from a day 13 2-AAF/CCl<sub>4</sub> rat. (A) BrdU staining of oval cells in the proliferative state. Very few cells are positively stained, which may represent the small peak of cells in the G2/M phase seen in Fig. 7C and 7D. The ductular formation (BD) is devoid of any BrdU staining. (B) Liver section stained for PCNA also shows little or no PCNA staining. This corroborates the BrdU staining. Both of these stains document that the majority of oval cells taken at this time point are not in a proliferative state. © and D) The corresponding positive controls for BrdU and PCNA staining on rat liver sections from the 24-hour Phx time point. *Arrows* indicate individual oval cells. (Original magnification [A-D] X100.)

FIGURE 14 A-H. Cytocentrifuged preparation of Thy1.1<sup>+</sup> sorted cells from 2-AAF/CCl<sub>4</sub>-treated rats. Thy-1.1<sup>+</sup> were stained with oval cell-specific antibodies. (A) Hematoxylin-eosin stain. (B) A representative of negative control (omission of primary antibody). (C-F) AFP, CK-19, GGT, and OV6 staining, respectively. The majority of the Thy-1<sup>+</sup> cells were positive for oval cell-specific markers. (G) A representative of cells stained for desmin. All photomicrographs are at 100X magnification. (H) Dual staining of oval cells. AFP-Texas Red (*red*) and Thy1.1<sup>+</sup>-FITC (*green*) showing both markers on the same cells. Where the two antibodies are in close proximity to each other, the wavelengths mix and the resulting fluorescence is yellow. Photomicrograph for (H) was obtained using confocal microscopy.

FIGURE 15 A-E. Cytocentrifuged preparation of Thy1.1<sup>+</sup> sorted cells from 2-AAF/CCl<sub>4</sub>-treated rats. The Thy-1<sup>+</sup> cells were also stained with oval cell antibodies. (A) Hematoxylin-eosin stain. (B and D) Cells stained for AFP and GGT, respectively. The corresponding negative controls are shown in (C) and (E). All photomicrographs are at 200X magnification.

FIGURE 16 A-F. (16A) Normal Rat Spleen. DPPIV positive cells with stain with a reddish/burn orange color. Notice that the white pulp region of the spleen is devoid of stain. 10x objective. (16B) Normal Rat Spleen. DPPIV positive

cells with stain with a reddish/burn orange color. Notice that the white pulp region of the spleen is devoid of stain. 20x objective. (16C) Experimental DPPIV deficient rat transplanted with DPPIV positive bone marrow. Spleen 60 days post BMTx. Notice the reddish/burn orange staining present throughout the spleen. The white pulp region is now showing a large number of cells positive for DPPIV. 4x objective. (16D) Experimental DPPIV deficient rat transplanted with DPPIV positive bone marrow. Spleen 60 days post BMTx. Notice the reddish/burn orange staining present throughout the spleen. The white pulp region is now showing a large number of cells positive for DPPIV. 10x objective.

FIGURE 17A-D. (17A) Normal Rat pancreas. DPPIV positive cells with stain with a reddish/burn orange color. 10x objective. (17B) Normal Rat pancreas. DPPIV positive cells with stain with a reddish/burn orange color. 20x objective. (17C) DPPIV deficient rat pancreas. No staining is visible throughout the section. 10x objective. (17D) DPPIV deficient rat pancreas. No staining is visible throughout the section. 20x objective. (17E) Experimental DPPIV deficient rat transplanted with DPPIV positive bone marrow. Pancreas 60 days post BMTx. Notice there are a few reddish/burn orange cells staining positive in the pancreas. 10x objective. (17F) Experimental DPPIV deficient rat transplanted with DPPIV positive bone marrow. Pancreas 60 days post BMTx. Notice there are a few reddish/burn orange positive cells present in the pancreas. 20x objective.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods and compositions for stimulating liver regeneration in a subject having a liver disorder. The invention provides methods and compositions for transplanting of bone marrow cells into a recipient host in amounts sufficient to result in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration.

In a specific embodiment of the invention, bone marrow cells are administered to a subject in need of new liver tissue. The bone marrow cells can be injected into the recipient, wherein the bone marrow cells will migrate to the liver, undergo proliferation, and differentiation leading to the production of new liver tissue

containing hepatocytes, bile ductal cells and oval cells. Alternatively, the bone marrow cells may be transplanted directly into the liver where the cells will proliferate and differentiate to form new liver tissue.

5 The compositions of the invention comprise bone marrow cells in a pharmaceutically acceptable carrier for administration into a recipient host in need of new liver tissue. The bone marrow cells may also be genetically engineered to enable them to produce a wide range of functionally active proteins, such as for example, growth factors, cytokines and hormones. The compositions of the invention also comprise bone marrow cells on a support matrix for transplantation into the liver. The  
10 matrix may further comprise growth factors capable of stimulating the proliferation and/or differentiation of hepatic stem cells or other types of cells.

The invention further relates to methods for enriching for populations of hepatic oval cells, a hepatic stem cell, using the Thy-1 cell surface antigen as an antibody tag. Once purified, the oval cells may be transplanted into a recipient in need  
15 of new liver tissue. The hepatic oval cells may be transplanted directly into the liver of the recipient where the hepatic oval cells will undergo proliferation, and differentiation leading to the production of new liver tissue containing hepatocytes, bile ductal cells and oval cells. Alternatively, the cells may be injected into the portal vein where the cells will go directly to the liver. In yet another embodiment of  
20 the invention, the hepatic oval cells may be injected into the spleen followed by migration to the liver.

### 5.1. SOURCES OF BONE MARROW CELLS

Bone marrow cells may be obtained from a variety of different donor sources. In a preferred embodiment, autologous bone marrow is obtained from the  
25 subject who is to receive the bone marrow cells. This approach is especially advantageous since the immunological rejection of foreign tissue and/or a graft versus host response is avoided. In yet another preferred embodiment of the invention, allogenic bone marrow may be obtained from donors who are genetically related to the recipient and share the same transplantation antigens on the surface of their blood

cells. Alternatively, if a related donor is unavailable, bone marrow from antigenically matched (identified through a national registry) donors may be used.

Bone marrow cells can be obtained from the donor by standard bone marrow aspiration techniques known in the art. For example, bone marrow cells can  
5 be removed from the donor by placing a hollow needle into the marrow space and withdrawing a quantity of marrow cells by aspiration. Alternatively, peripheral stem cells can be obtained from a donor, for example, by standard phlebotomy or apheresis techniques. For convenience, the following embodiments of the invention are described for bone marrow cells, although it should be understood that peripheral stem  
10 cells may be used as equivalent to bone marrow cells.

Before administration into the recipient, bone marrow cell populations maybe enriched for stem cells by selecting for cells that express stem cell surface antigens such as Thy-1, CD34, *Flt-3* ligand and *c-kit*, in combination with purification techniques such as immuno-magnetic bead purification, affinity chromatography and  
15 fluorescence activated cell sorting. In addition, where the possibility of a graft versus host response exists, the stem cells to be administered to the recipient can be T-cell depleted to prevent the development of a graft versus host response. The cell population maybe depleted of T-cells by one of many methods known to one skilled in the art (e.g., Blazer et al., 1985, *Experimental Hematology* 13:123-128).

Prior to transplantation into the recipient host, the bone marrow cells may be stimulated with a number of different growth factors that can regulate tissue regeneration by affecting cell proliferation, differentiation and gene expression. Such growth factors include those capable of stimulating the proliferation and/or  
20 differentiation of bone marrow cells and hepatic progenitor cells. For example, epidermal growth factor (EGF), transforming growth factor  $\alpha$  (TGF- $\alpha$ ) or hepatocyte growth factor/scatter factor (HGF/SF) may be utilized. The bone marrow cells may be stimulated *in vitro* prior to transplantation into the recipient subject. Alternatively, the stem cells may be stimulated *in vivo* by injecting the recipient with such growth  
25 factors following transplantation.

30 The present methods and compositions can also employ bone marrow cells genetically engineered to enable them to produce a wide range of functionally

active biologically active proteins, including but not limited to growth factors, cytokines, hormones, inhibitors of cytokines, peptide growth and differentiation factors. Methods which are well known to those skilled in the art can be used to construct expression vectors containing a nucleic acid encoding the protein coding  
5 region of interest operatively linked to appropriate transcriptional/translational control signals. See, for example, the techniques described in Sambrook, et al., 1992, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, N.Y.

10 In addition, stem cells may be attached *in vitro* to a natural or synthetic matrix that provides support for the transplanted cells prior to transplantation. The type of matrix that may be used in the practice of the invention is virtually limitless. The matrix will have all the features commonly associated with being “biocompatible”, in that it is in a form that does not produce an adverse, or allergic  
15 reaction when administered to the recipient host. Growth factors capable of stimulating the growth and regeneration of liver tissue may also be incorporated into the matrices. Such matrices may be formed from both natural or synthetic materials and may be designed to allow for sustained release of growth factors over prolonged periods of time. Thus, appropriate matrices will both provide growth factors and also  
20 act as an *in situ* scaffolding in which the transplanted cells differentiate and proliferate to form new liver tissue. In preferred embodiments, it is contemplated that a biodegradable matrix that is capable of being reabsorbed into the body will likely be most useful.

To improve stem cell adhesion to the matrix, and survival and function  
25 of the stem cell, the matrix may optionally be coated on its external surface with factors known in the art to promote cell adhesion, growth or survival. Such factors include cell adhesion molecules, extra cellular matrix molecules and/or growth factors.

The present invention also relates to the use of bone marrow cells in  
30 three dimensional cell and tissue culture systems to form structures analogous to liver tissue counterparts *in vivo*. Cells cultured on a three-dimensional culture system will

grow in multiple layers, forming new liver tissue. The resulting liver tissue will survive for prolonged periods of time, and perform liver-specific functions following transplantation into the recipient host. Methods for producing such structures is described, *e.g.*, in US Patent No. 5,624,840, which is incorporated herein in its entirety.

## 5.2 METHODS FOR ENRICHMENT OF HEPATIC OVAL CELLS

The present invention also provides methods for deriving an enriched population of hepatic oval cells from liver tissue using a Thy-1 specific antibody. This aspect of the invention is based on the observation that hepatic oval cells express high levels of Thy-1 on their cell surface.

Hepatic oval cells may be obtained from a variety of different donor sources. Depending on the degree of liver damage, enriched populations of autologous hepatic oval cells may be derived from the tissue of the subject who is to receive the transplanted hepatic oval cells. This approach avoids the immunological rejection of foreign tissue. In yet another preferred embodiment of the invention, allogenic liver tissue for use in purifying hepatic oval cells may be obtained from donors who are genetically related to the recipient and share the same transplantation antigens on the surface of their blood cells. Alternatively, if a sibling is unavailable, tissue may be derived from antigenically matched (identified through a national registry) donors.

In an embodiment of the invention, hepatic oval cells are isolated from a disaggregated liver tissue biopsy. This may be readily accomplished using techniques known to those skilled in the art. For example, the liver tissue can be disaggregated mechanically and/or treated with digestive enzymes and/or chelating agents that weaken the connections between neighboring cells, making it possible to disperse the tissue suspension of individual cells. Enzymatic dissociation can be carried out by mincing the liver tissue and treating the minced tissue with any of a number of digestive enzymes. Such enzymes include, but are not limited to, trypsin, chymotrypsin, collagenase, elastase and/or hyaluronidase. A review of tissue disaggregation technique is provided in, *e.g.*, Freshney, Culture of Animal Cells, A



Manual of Basic Technique, 2d Ed., A.R. Liss, Inc., New York, 1987, Ch. 9, pp.107-126.

Following preparation of a single cell suspension, Thy 1.1-positive cells, which represent the hepatic oval cell population of cells may be purified from the Thy1-1-negative population of cells using a variety of different methods. Such procedures involve a positive selection, such as passage of sample cells over a column containing anti-Thy-1 antibodies or binding of cells to magnetic bead conjugated anti-Thy1 antibodies or by panning on anti-Thy-1 antibody coated plates and collecting the bound cells. Alternatively, the single cell suspension may be exposed to a labeled antibody that immuno-specifically binds to the Thy1-1 cell surface antigen. Following incubation, with the Thy 1.1 antibody, the cells are rinsed in buffer to remove any unbound antibody. Hepatic oval cells expressing the Thy1-1 cell surface antigen can then be cell sorted by fluorescence-activated cell sorting using, for example, a Becton Dickinson FACStar flow cytometer.

Prior to transplantation into the recipient host, the hepatic oval cells may be contacted with a number of different growth factors that can regulate tissue regeneration by affecting cell proliferation, and gene expression. Such growth factors include those capable of stimulating the proliferation and/or differentiation of hepatic progenitor cells. For example, epidermal growth factor (EGF), transforming growth factor  $\alpha$  (TGF- $\alpha$ ) or hepatocyte growth factor/scatter factor (HGF/SF) may be utilized. The hepatic oval cells may be stimulated *in vitro* prior to transplantation into the recipient subject, or alternatively, by injecting the recipient with growth factors following transplantation.

The present methods and compositions may employ hepatic oval cells genetically engineered to enable them to produce a wide range of functionally active biologically active proteins including, but not limited to, growth factors, cytokines, hormones, inhibitors of cytokines, peptide growth and differentiation factors. Methods which are well known to those skilled in the art can be used to construct expression vectors containing a nucleic acid encoding the protein of interest linked to appropriate transcriptional/translational control signals. See, for example, the techniques described in Sambrook, et al., 1992, Molecular Cloning, A Laboratory

Manuel, Cold Spring Harbor Laboratory, N.Y., and Ausebel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, N.Y.

5 In addition, hepatic oval cells may be attached *in vitro* to a natural or synthetic matrix that provides support for the transplanted hepatic oval cells prior to transplantation. The matrix will have all the features commonly associated with being “biocompatible”, in that it is in a form that does not produce an adverse, or allergic reaction when administered to the recipient host. Growth factors capable of stimulating the growth and regeneration of liver tissue may also be incorporated into  
10 matrices. Such matrices may be formed from both natural or synthetic materials and may be designed to allow for sustained release of growth factors over prolonged periods of time. Thus, appropriate matrices will both provide growth factors and also act as an *in situ* scaffolding in which the hepatic oval cells differentiate and proliferate to form new liver tissue. In preferred embodiments, it is contemplated that a  
15 biodegradable matrix that is capable of being reabsorbed into the body will likely be most useful.

To improve oval cell adhesion to the matrix, and survival and function of the stem cell, the matrix may optionally be coated in its external surface with factors known in the art to promote cell adhesion, growth or survival. Such factors  
20 include cell adhesion molecules, extra cellular matrix molecules or growth factors.

The present invention also relates to the use of hepatic oval cells in three dimensional cell and tissue culture systems to form structures analogous to liver tissue counterparts *in vivo*. The resulting liver tissue will survive for prolonged periods of time, and perform liver-specific functions following transplantation into the recipient host. Methods for producing such structures is described in US Patent No.  
25 5624,840, which is incorporated herein in its entirety.

### 5.3. ADMINISTRATION OF BONE MARROW

#### STEM CELLS OR HEPATIC OVAL CELLS

The bone marrow cells and/or enriched oval cells can be administered  
30 to the recipient in an effective amount to achieve its intended purpose. More

specifically, an effective amount means an amount sufficient to lead to the development of new liver tissue and restoration of liver function, thereby alleviating the symptoms associated with liver disorders.

The number of cells needed to achieve the purposes of the present invention will vary depending on the degree of liver damage and the size, age and weight of the host. For example, the cells are administered in an amount effective to restore liver function. The dose range of cells to be used in the practice of the invention may vary between  $10^5$  -  $10^{10}$  cells, although the preferable dose of administered cells will be between  $10^6$  -  $10^8$ . It may be necessary to use dosages outside these ranges in some cases, as will be apparent to those of skill in the art.

Determination of effective amounts is well within the capability of those skilled in the art. The effective dose may be determined by using a variety of different assays designed to detect restoration of liver function. The progress of the transplant recipient can be determined using assays that include blood tests known as liver function tests. Such liver function tests include assays for alkaline phosphates, alanine transaminase, aspartate transaminase and bilirubin. In addition, recipients can be examined for presence or disappearance of features normally associated with liver disease such as, for example, jaundice, anemia, leukopenia, thrombocytopenia, increased heart rate, and high levels of insulin. Further, imaging tests such as ultrasound, computer assisted tomography (CAT) and magnetic resonance (MR) may be used to assay for liver function.

The bone marrow cells and/or enriched oval cells can be administered to the recipient in one or more physiologically acceptable carriers. Carriers for these cells may include, but are not limited to, solutions of phosphate buffered saline (PBS) containing a mixture of salts in physiologic concentrations. In addition, the cells may be associated with a matrix prior to administration into the recipient host.

In an embodiment of the invention, the bone marrow cells and/or hepatic oval cells can be administered by intravenous infusion. The cells to be injected, are drawn up into a syringe and injected into the recipient host. In such

instances the cells would be expected to migrate to the recipient's liver where they will differentiate and proliferate to form new liver tissue.

Alternatively, the methods of the present invention encompass administration of the bone marrow cells and/or hepatic oval cells into the recipient so as to become located in the liver. The administration of the stem cells and/or hepatic oval cells, is accomplished by conventional techniques such as injection of cells into the recipient host liver, injection into the portal vein, or surgical transplantation of cells into the recipient host liver. In some instances it may be necessary to administer the stem cells and/or hepatic oval more than once to restore liver function. In addition, growth factors, such as G-CSF, or hormones, may be administered to the recipient prior to and following transplantation for the purpose of priming the recipients liver and blood to accept the transplanted cells and/or to generate an environment supportive of hepatic cell proliferation.

#### 5.4. USE OF BONE MARROW CELLS FOR REGENERATION OF TISSUE, OTHER THAN LIVER TISSUE

In yet another embodiment of the invention, bone marrow cells may be used for regeneration of tissues other than liver tissue. Specifically, the methods and compositions of the invention provide for the transplantation of bone-marrow stem cells into a recipient host in amounts sufficient to result in tissue regeneration other than liver regeneration. The invention is based on the observation that bone-marrow derived cells can participate in the production of not only liver cells, but pancreatic cells as well.

The methods of the present invention encompass administration of the bone marrow cells into the recipient host so as to become located in the organ or tissue in which regeneration is desired. The administration of the stem cells into the desired region is accomplished by conventional techniques such as injection of cells within the recipient host or surgical transplantation of cells within the recipient host. In some instances it may be necessary to administer the stem cells more than once to restore the desired tissue function.

5 administered to the recipient in one or more physiologically acceptable carriers, in an effective amount to achieve its intended purpose. More specifically, an effective amount means an amount sufficient to lead to the development of new pancreatic tissue and restoration of pancreatic function, thereby alleviating the symptoms associated with the pancreatic disorders.

10            6.        EXAMPLE: BONE MARROW CELLS PARTICIPATE IN THE  
                         PRODUCTION OF HEPATOCYTES, BILE DUCTAL  
                         CELLS AND OVAL CELLS DURING LIVER  
                         REGENERATION

cells and other liver cells may arise from a cell population originating in, or associated with, the bone marrow. This hypothesis was tested by three approaches: i) bone marrow transplantation (BMTx) from male rats into lethally irradiated syngeneic females, and detection of donor cells in the recipients by means of DNA probes to the Y chromosome *sry* region; ii) BMTx from Dipeptidyl peptidase-IV positive (DPPIV<sup>+</sup>) male rats into DPPIV<sup>-</sup> syngeneic females, and detection of DPPIV-expressing cells in the recipient animals; and iii) whole liver transplantation (WLTx) using Lewis rats that express the L21-6 antigen as recipients, and Brown-Norway (Brown-Norway) rats that do not express this antigen as allogenic donors, in order to confirm that an extra-hepatic source (L21-6<sup>+</sup> cells) could repopulate the transplanted (L21-6<sup>-</sup> cells) liver. In conjunction with these approaches, the 2-AAF/CCl<sub>4</sub> protocol was used to induce oval cell activation and proliferation. Hepatocyte proliferation is generally suppressed by exposure of an animal to 2-acetylaminoflourane (2-AAF) and subsequently hepatic injury is induced by administration of carbon tetrachloride. *In situ* hybridization, PCR,

and immunohisto- and cytochemical techniques were used to distinguish donor cells from recipient cells.

## 6.1. MATERIALS AND METHODS

### 6.1.1. BONE MARROW AND WHOLE LIVER TRANSPLANTATION

5 Bone marrow transplantation (BMTx) was performed as previously described (Murase, N. et al., 1996, Transplantation 61:1) with minor modifications. In one set of experiments (Set 1, two separate experiments), bone marrow from male F-344 rats was transplanted into lethally irradiated syngeneic female F-344 rats (n = 10 animals). The females were given 10.5 Gy from a <sup>137</sup>Cesium source (JL Shepherd Mark I), and then rescued by injecting about 60 x 10<sup>6</sup> male bone marrow cells via the tail vein. After allowing establishment of a chimeric system (about 30-45 days), the animals were tested to see if the donor cells had engrafted. PCR analysis was performed on DNA extracted from the buffy coat of nucleated cells obtained from retinal orbital blood (ROB). All animals survived the transplant procedure, but  
10 varying degrees of intensity for chimerism, was evident. Only those animals that expressed a strong signal for the Y chromosome PCR product were placed on the 2-AAF/CCl<sub>4</sub> protocol for oval cell induction. The animals were sacrificed 9-13 days after CCl<sub>4</sub> administration. In a second set of BMTx experiments (Set 2), bone marrow from male F-344 rats expressing the DPPIV enzyme was transplanted into  
15 lethally irradiated DPPIV<sup>-</sup> syngeneic female F-344 rats, which were then further treated as above. Ten female rats were successfully transplanted in Set 1, and fourteen female rats in Set 2.

Whole liver transplantation (WLTx) was performed as previously described (Murase, N et al., 1995, Transplantation 60:158). Brown-Norway rats were  
25 used as donors of whole liver tissue, and Lewis rats as recipients. The L21-6 monoclonal antibody specific for Lewis rats was used to distinguish immunohistochemically donor cells from recipient cells (Yagihashi et al., 1995, Transplantation Proceedings 27:1519). Once the rats recovered from the WLTx (about two months), the animals were placed on the 2-AAF/CCl<sub>4</sub> protocol for  
30 induction of oval cells as described below. In this experiment six Lewis rats were

successfully transplanted; of these, three animals survived until completion of the study.

#### 6.1.2. ANIMALS

5 F-344 male and female rats were obtained from Frederick Laboratories (Frederick, MD) as marrow donors and recipients. DPPIV<sup>-</sup> female F-344 rats were a gift from Dr. Sanjeev Gupta, Albert Einstein College of Medicine, Bronx, NY. All procedures involving animals were conducted according to institutionally approved protocols.

#### 6.1.3. INDUCTION OF OVAL CELLS

10 2-AAF time-release pellets were prepared (70 mg, 2.5 mg/day release for 28 days) as described by Hixson et al. (Hixson D.C. et al., 1990, Pathobiology 58:65) and inserted subcutaneously in the rats 7 days prior to administration of CCl<sub>4</sub>. The animals were sacrificed, and tissue samples were obtained at various time points thereafter. CCl<sub>4</sub> was injected intraperitoneally (i.p) as a single dose of 1.9 ml/kg  
15 (1500 mg/kg) b.w. of a 1: 1 (vol/vol) solution in corn oil; this dose was calculated on the basis of the LD<sub>50</sub> dose (RJ. Lewis, Sr., Ed., *SAX Dangerous Properties of Industrial Material* (Van Nostrand Reinhold, New York, NY. 1993), pp. 52 and 1149, eighth edition).

#### 6.1.4. PCR ANALYSIS

20 PCR analysis for the Y chromosome was performed on DNA extracted from transplanted and non-transplanted female animals using primers for the *sry* gene of the Y chromosome (An, J. et al., 1997, J Andrology 18:289). The primer sequences are as follows:

5'-CATCGAAGGGTTAAAGTGCCA-3' and

25 5'-ATAGTGTGTAGGTTGTTGTCC-3'. These primers amplify a 549-bp nucleic acid product that has been shown to be very specific for the Y chromosome with no reactivity to female DNA. PCR was performed as previously described (An, J. et al., 1996, Experimental Hematology 24:768). Human  $\beta$ -actin primers from Clontec Laboratories (Palo Alto, CA), included to ensure that each sample of DNA was intact,

produce an expected product of approximately 1000 base pairs in length (DeFrances, M.C., 1992, Development 116:387).

#### 6.1.5. CELL ISOLATION

Hepatocytes and nonparenchymal cells (NPC) were isolated after a 2-step collagenase digestion of the liver according to an established protocol (1996, Methods Cell Biol 13:29) Prior to the isolation procedure, the caudate lobe was surgically removed and divided into halves, one being fixed in 10% buffered formalin, and the other placed in OTC. compound and frozen in cold 2-Methylbutane. The samples were stored at -80°C until paraffin or frozen sections were prepared for routine examination after hematoxylin and eosin (H&E) staining. This tissue served as an internal control for light microscopy and hybridization *in situ*.

Digestion began by blanching the liver to remove the majority of blood cells from the liver, by perfusion with a buffered saline solution (S&M) for 10 min, 10 ml/min at 37°C. Digestion of the liver was accomplished by collagenase digestion (Worthington Biochemical Co., Freehold, NJ; 100 mg/250 ml S&M supplemented with CaCl) for 20 min, at 10 ml/min at 37°C. On completion of the digestion, the liver was removed from the animal, placed into cold S&M, and repeatedly shaken to disrupt the individual cells from the tissue. The resulting cell suspension was passed through nylon mesh and centrifuged 3 times at 50 g for 5 min to separate the hepatocytes from the NPCs. The supernatants on top of the hepatocyte pellets were collected after each spin and combined. The hepatocytes were resuspended in cold media (MEM Neaa, Gibco, Gaithersburg, MD) and placed on ice until further use. The collected supernatant was diluted 1:3 with cold S&M to remove the collagenase from the cells, and centrifuged at 1100 rpms (400 g) for 10 min to pellet the NPCs. The NPC fraction was then suspended in 1X phosphate buffered saline (PBS) and stored at 4°C until oval cell isolation. The presence of oval cells in the NPC fraction has been previously determined (Yaswen, P., 1984, Cancer Research 44:324). The oval cells were then isolated from the NPC fraction using flow cytometric techniques as previously described (Petersen, B.E. et al., 1998, Hepatology 27:433). Briefly, approximately 200 x 10<sup>6</sup> NPCs were incubated for 20 min at 4°C with fluorescein



isothiocyanate (FITC)-conjugated anti-rat Thy 1.1 (1  $\mu$ g/million cells) and then rinsed twice in 1X PBS + 1%FBS, 5 min each time. FITC isotype mouse IgG<sub>1</sub> was used as control. The cells were then kept on ice in the dark until sorted with a Becton Dickinson FACStar flow cytometer into Thy1.1<sup>+</sup> (oval cells) and Thy1.1<sup>-</sup> sub-  
5 populations of cells. The purity of the different sorted cell populations were as follow: 95-97% pure for the Thy-1<sup>+</sup> cells and 99% pure for the Thy-1<sup>-</sup> cells (Petersen, B.E. et al., 1998, Hepatology 27:433).

#### 6.1.7. *IN SITU* HYBRIDIZATION OF THE *SRY* REGION OF THE Y CHROMOSOME

10 Digoxigenin labeled DNA probes, prepared by random priming using the Genius System instructions (Boehringer Mannheim, Indianapolis, IN), were hybridized to paraffin or frozen liver sections as per manufacturer's instructions. Briefly, frozen sections were placed on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and were pre-hybridized in 10 mM Tris-HCl, 50%  
15 formamide, 0.6 M NaCl, 1 mM EDTA, 1x Denhardts, 0.5 mg/ml carrier RNA and 10% dextran sulfate for 1 hr at 37°C. The digoxigenin-labeled probe of the *sry* gene of the Y chromosome was applied to the sections and allowed to hybridize overnight at 37°C. Following post-hybridization washes, detection of the probe was accomplished by incubation with alkaline phosphatase-conjugated anti-digoxigenin  
20 antibody (1:500) for 2 hr at RT. Alkaline phosphatase activity was visualized by incubation with NBT and BICP (Boehringer Mannheim, Indianapolis, IN) in the dark. The color development was monitored and the enzymatic reaction stopped by immersing the slides in 10 mM Tris, 1 mM EDTA.

#### 6.1.8. IMMUNOHISTOCHEMISTRY AND CYTOCHEMISTRY

25 All tissue samples were divided into half. One was immediately placed in liquid nitrogen for later extraction of RNA or DNA. The other half was split, with one portion being fixed in 10% buffered formalin and processed for preparation of 4- $\mu$ m thick paraffin embedded sections, and the other portion placed in OTC freezing compound and cold 2-Methylbutane and processed for preparation of

6  $\mu$ m-thick frozen sections which were stored at -80°C until staining. Routine histological examinations were performed on both paraffin and frozen sections stained with hematoxylin and eosin (H&E). In addition, spleen and pancreas tissue were treated the same way and frozen sections and paraffin sections were DPPIV and  
5 hematoxylin and eosin stained.

## 6.2. RESULTS

Female rats were lethally irradiated and rescued with a bone marrow transplant from a male animal. Nucleated blood cells of the transplanted animals were tested by PCR to establish that the BMTx was successful. Once the female rats were  
10 determined to have been engrafted with male bone marrow, they were placed on the oval cell protocol as stated above. On Day 9 and Day 13 (post hepatic injury) rats were anesthetized and their livers were perfused in order to obtain single cell suspensions of non-parenchymal cells (NPC) (cells that are not hepatocytes) and parenchymal cells (primarily hepatocytes).

15 Flow cytometry was used to isolate the oval cells from the NPC fraction using anti-Thy-1-FITC. The Thy-1 positive, Thy-1 negative and hepatocyte populations were subjected to PCR analysis. Figure 1 shows that both the day 9 and day 13 Thy-1<sup>+</sup> and Thy-1<sup>-</sup> cell populations of NPCs were positive for the Y chromosome PCR product. The Thy-1<sup>-</sup> fraction showed a strong signal, probably  
20 due to the presence of donor hematopoietic cells such as Kupffer cells which are in the NPC population and Thy-1 negative, but positive for the Y chromosome. In the day 9 hepatocyte fraction there was no visible signal. At this time point in oval cell activation, the oval cells have not yet begun to differentiate into either transitional cells or hepatocytes. By day 13 there were cells in the hepatocyte fraction expressing  
25 the Y chromosome PCR (549 bp) product. At this time the oval cells are beginning to differentiate into hepatocytes (Grishman, J.W., 1997, in Stem Cells, C.S. Potten, Ed. (Academic Press, San Diego, CA p233-282). If all oval cells that differentiate into hepatocytes were derived from the liver, then one would expect that none of the hepatocytes tested would be positive for the Y chromosome. The finding that some  
30 hepatocytes were Y chromosome positive indicates that they were derived from the

bone marrow donor cells. The combined data indicates that at day 9 the oval cells (Thy-1<sup>+</sup>) in the recipient female were derived from the donor male and that they continued to differentiate into mature hepatocytes by day 13.

To confirm the PCR results seen in Figure 1, one of the smaller lobes from every liver that was perfused was ligated, removed and split in half. One portion was fixed in 10% buffered formalin and processed into 4- $\mu$ m thick paraffin embedded sections; the other portion was placed in OTC freezing compound and processed into 6  $\mu$ m-thick frozen sections. *In situ* hybridization for the Y chromosome *sry* gene was performed on both types of fixed tissue. Hepatocytes carrying a positive reaction product (blue staining) in their nuclei were readily seen in untreated control male rats (Figures 2A). In agreement with the results obtained by PCR analysis of the isolated hepatocyte fraction, cells with positive blue staining (Y chromosome positive) were detected in females subjected to BMTx and the 2-AAF/CCl<sub>4</sub> protocol at day 13 (Figure 2B). Figure 2C shows no reaction product in the liver of an untreated control female.

In the second set of bone marrow transplantation experiments, bone marrow cells from DPPIV<sup>+</sup> F-344 male rats were injected into lethally irradiated DPPIV<sup>-</sup> F-344 females. This constituted a system in which the presence of cells originating from donor cells in the recipient liver could be easily detected, by revealing cytochemically the activity of the enzyme DPPIV (Figure 3). As previously reported with studies of this enzyme, a diffuse red to brownish-red staining of the bile canalicular site between hepatocytes was observed. This type of staining was seen in the DPPIV<sup>+</sup> F-344 male rats (Figure 3A). The control untreated DPPIV<sup>-</sup> females showed no staining (Figure 3B). To determine whether DPPIV would be expressed in transplanted animals, staining was performed on liver sections prepared from transplanted female rats treated with 2-AAF/CCl<sub>4</sub>. As seen in Figure 3C-3F, DPPIV expression was observed in several bile canalicular sites between hepatocytes from four different transplanted animals. DPPIV expression was also observed on a few oval cells/transitional (small hepatocyte) cells in the liver from these rats (Figure 3D and 3E).

Four animals showing the strongest evidence of the donor marrow engraftment, based upon examination of the recipient rat's spleens, were chosen

(Figure 3C-3F) for an estimation of the number of hepatocytes originating from the donor bone marrow. From these four animals, five different lobes were used to cut approximately 10 sections per lobe (50 sections total). All of the sections were stained for DPPIV and examined for DPPIV expression. Roughly, 25 random fields (200x) per section were examined, and the numbers indicate the total number of hepatocytes positive for DPPIV staining per section.

Table 1 represents the number of DPPIV positive hepatocytes observed in the transplanted DPPIV<sup>-</sup> rats. By dividing the total positive cells by the total hepatocytes observed, approximately 0.16% of the total number of hepatocytes were positive for DPPIV expression. The rat liver has approximately  $700 \times 10^6$  hepatocytes, indicating that, at day 13, approximately  $1.0 \times 10^6$  hepatocytes originated from transplanted bone marrow cells using the oval cell protocol.

15

TABLE 1. Percent of DPPIV positive hepatocytes in BMTx DPPIV deficient females					
Animal Number	LOBES # of Different	SECTIONS # of Different non-serial	FIELDS Total # of Random Fields	DPPIV+ Total # of positive Hepatocytes	PERCENT TOTAL
6028	5	10	250	55	0.144
6034	4	10	200	18	0.059
6036	5	10	250	78	0.204
6037	5	10	250	93	0.243

The mean value of 0.163% represents that approximately  $1 \times 10^6$  hepatocytes would be positive for DPPIV expression within the transplanted female rats.

In order to confirm that extra-hepatic cells can repopulate the liver, whole liver transplantation was used as a final approach. In this series of experiments, Lewis rats that express the MHC class II L21-6 isozyme were used as recipients of liver from Brown-Norway rats which do not express L21-6 (Yagihushi, A. et al., 1995, Transplantation Proceedings 27:1519). An anti L21-6 monoclonal antibody was used to differentiate donor from recipient cells. In this model, oval cells that

originated from an extra-hepatic source would be L21-6 positive, while oval cells originating *in situ* would be negative. The results obtained in these experiments are illustrated in Figures 4 and 5. Figure 4A and 4B shows stained sections of two Brown-Norway rat livers transplanted into Lewis rats prior to being placed on the 2-AAF/CCl<sub>4</sub> protocol. A widespread staining of L21-6 was present in the transplanted Brown-Norway livers, presumably due to influx of cells of the Lewis host immune system reacting to the allogenic Brown-Norway liver. Most notable, though, was the presence of ductal structures containing L21-6 positive cells. These structures represent a pattern often seen in the organization and differentiation of actively proliferating oval cells (Thorgeirsson, S.S. et al., 1993, Proc. Soc. Exp Biol Med 204:253; N. Fausto, in *The Liver: Biology and Pathobiology* I.M. Arias et al. Eds (Raven Press, New York 1994) p. 1501-1518 (third edition)). The presence therein of positive cells supports the concept that these oval cells were derived from an extra-hepatic source. The structures also contained cells which were clearly L21-6 negative, suggesting that some oval cells were derived *in situ* from the donor liver (Figure 4B).

To better characterize the positive cells seen in the ductal structures and distinguish them from inflammatory cells invading the Brown-Norway transplanted livers, double immunofluorescence staining was performed using an antibody against OC.2, a specific oval cell/ductal cell marker (Faris, R.A. et al., 1956 Cancer Research 16:142), in conjunction with the L21-6 antibody. As can be seen in Figure 5, individual cells expressing both OC.2 and L21-6 are evident as a yellow stain (combination of red and green). The cells, therefore, were identified as oval cells or derivatives thereof, and not immunocytes or inflammatory cells.

In addition, when tissues other than the liver were analyzed, such as the pancreas and spleen, and cell staining was observed, indicating that transplanted bone marrow cells were capable of infiltrating and incorporating into these tissues. As indicated in Figure 17A-F, when experimental DPPIV deficient rats were transplanted with DPPIV positive bone marrow, a large number of cells were positive for DPPIV (Figure 17 E-F).

7. EXAMPLE: PURIFICATION OF HEPATIC OVAL  
CELLS USING ANTIBODIES THAT RECOGNIZE THE  
THY1.1 CELL SURFACE ANTIGEN

The following subsection discloses experimental data indicating that  
5 hepatic oval cells express the hematopoietic stem cell marker Thy1.1. The data  
provides a novel cell marker for identification of oval cells. Using Thy1.1 antibody, a  
highly enriched population of oval cells was obtained.

7.1 MATERIALS AND METHODS

CCl<sub>4</sub>, 99% pure high-performance liquid chromatography grade, and 2-  
10 AAF were purchased from Aldrich Chemical Co. (St. Louis, MO). 2-AAF crystals  
were incorporated into time-released pellets (70 mg/pellet over a 28-day release, 2.5  
mg/d) supplied by Innovative Research Inc. (Sarasota, FL). Male Fisher 344 rats  
(150-170 g) were obtained from Frederick's Laboratories (Frederick, MD).  
Microscope Superfrost Plus slides, buffered Formalin-Fresh, and dextran sulfate were  
15 obtained from Fisher Scientific (Pittsburg, PA). Hematoxylin was purchased from  
Anatech, Ltd. (Battle Creek, MI). Anti- $\alpha$ -fetoprotein (AFP) antibody was purchased  
from Nordic Immunology (Tilburgh, the Netherlands). OV-6 and BD-1 antibodies  
were gifts from Dr. Doug Hixson (Brown University, Providence, RI). OC.2 antibody  
was a gift from Dr. Ron Faris (Brown University, Providence, RI). Rat anti-GGT was  
20 a gift from Dr. Benito Lombardi (University of Pittsburgh, Pittsburgh, PA). Thy1.1  
was purchased from PharMingen Inc. (San Diego, CA). Proliferating cell nuclear  
antigen (PCNA) was purchased from Signet Laboratory Inc. (Dedham, MA). 5-  
Bromodeoxy- uridine (BrdU) was obtained from Boehringer Mannheim (Indianapolis,  
IN). Desmin was obtained from Dako Corp. (Carpinteria, CA). Eosin, propidium  
25 iodide (PI), and all other chemicals used were obtained from Sigma Chemical  
Company (St. Louis, MO).

### 7.1.2 COMPOUND DELIVERY

An LD<sub>50</sub> dose of CCl<sub>4</sub> as determined by Lewis was used (SAX Dangerous Properties of industrial Material, In: Lewis, R.J., Sr. Edition 8, 1993 update and vol 3. New York: Van Nostrand Reinhold, 1992 52:1149). A single dose  
5 of 1.9 mL/kg (1,500 mg/kg) of body weight, in a 1:1 vol/vol dilution in corn oil, was administered by intraperitoneal injection. Two hours before they were sacrificed, the animals received an intra peritoneal injection of BrdU (50 mg/kg body weight) to identify cells in S-phase of the cell cycle, as described by Lindrose et al (1991, Hepatology 13:743-750).

### 10 7.1.3 OVAL CELL COMPARTMENT PROLIFERATION/ACTIVATION

2-AAF pellets were inserted 7 days before hepatic injury following a protocol similar to Novikoff et al. (1996, Am J Pathol 148:1473-1491) and Hixson et al. (1990, Pathobiology 58:65-73) The time points for this study were counted from  
15 when the hepatic injury (CCl<sub>4</sub>, Phx) was induced. The dose and delivery for CCl<sub>4</sub> was discussed earlier in the compound delivery section and performed in the same manner. For the Phx procedure, rats were hepatectomized under general anaesthesia according to the methods described by Higgins and Anderson (1931, Arch. Pathol. 12:186-202). The tissue obtained was processed in the same manner described in the immunohistochemistry methods.

### 20 7.1.4 ANIMAL EUTHANIZATION

All procedures involving animals were conducted according to institutionally approved protocols. Rats were anesthetized by injection with sodium pentobarbital (0.1 mL/100 g body weight) before being sacrificed.

### 25 7.1.5 IMMUNOHISTOCHEMISTRY

A basic immunohistochemical protocol previously described by Wolf et al. (1991, Hepatology 12:186-202) was used with slight modification to conform to each particular antibody. Liver tissue was divided and fixed in either 10% buffered formalin or placed in OTC compound, frozen in cold 2-methylbutane (Fisher

Scientific), and stored at  $-80^{\circ}\text{C}$ . All staining procedures for light microscopy were performed on  $4\text{-}\mu\text{m}$  thick, paraffin-embedded sections or  $6\text{-m}$  thick, frozen sections. Routine histological examination were made for all liver tissue samples on sections (paraffin and frozen) stained with hematoxylin-eosin. Single cell suspensions were collected on glass slides by cytocentrifugation and air-dried. Cytocentrifugation was performed using a Cytospin 3 Cytocentrifuge (Shandon Inc. Pittsburgh, PA) for 6 minutes at 600 rpm. Immunohistochemistry on cytopsin preparations (100,000 cells/slide) was performed using the techniques described above. The cytopsin preps were then analyzed by confocal microscopy (Multiprobe 2001 Inverted Confocal Laser Scanning Microscope, CLSM, Molecular Dynamics, Sunnyvale, CA). BrdU staining was performed on  $4\text{-}\mu\text{m}$  thick, paraffin-embedded tissue as described by Lindroos et al. (1991, Hepatology 12:186-202). For each, antibody-negative controls were performed by either blocking with the appropriate nonimmune serum or by omitting the primary antibody from the protocol.

#### 15 7.1.6. FLOW CYTOMETRY

Hepatocyte and nonparenchymal cell isolation was performed by a two-step collagenase digestion according to the protocol established by Seglen (1976, Methods in Cell Biol 13:29-83). Oval cell isolation was performed using flow cytometry. Briefly, the nonparenchymal cell (NPC) fraction has been determined to contain the hepatic oval cell population as described by Yaswen et al. (1984, Cancer Res 44:324-331). Immunohistochemistry was performed on the parenchymal and NPC fractions to ensure that the cells of interest were in the NPC fraction. The NPC fraction was found to contain the highest percentage of oval cells. A portion (approximately  $60$  to  $80 \times 10^6$  of the total  $200 \times 10^6$  cells) of the NPC fraction was further purified using flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated anti-rat Thy1.1 (1 mg/million cells) was used to label the target cells. The cell fraction was incubated with the antibody for 20 minutes at  $4^{\circ}\text{C}$ , rinsed twice in  $1\times$  phosphate-buffered saline + 1% fetal bovine serum for 5 minutes each, and stored in the dark on ice until sorting. A FITC mouse  $\text{G}_1$  IgG<sub>1</sub> was used as an isotype control. Cells were sorted using a Becton Dickinson FACStar flow cytometer into two



populations: Thy1.1-positive (Thy1.1<sup>+</sup>) and Thy1.1-negative (Thy1.1<sup>-</sup>). Those animals whose livers were perfused had one of the smaller lobes surgically removed before the cell isolation procedure. This tissue was used as an internal control for light microscopy, as well as for *in situ* hybridization. The excised liver tissue was divided in half and fixed in either 10% buffered formalin or placed in OTC compound, frozen in cold-2-methylbutane (Fisher Scientific), and stored at -80°C.

*Cell Cycle Analysis.* One x 10<sup>6</sup> Thy1.1<sup>+</sup> cells were fixed in ice-cold 70% ethanol at 4°C overnight. Following centrifugation (5 minutes at 3,000 rpm), the ethanol was removed and 1 mL of propidium iodide staining solution consisting of 50 mg/mL PI, 100 U/mL RNase A in Ca- and Mg-free phosphate-buffered saline + glucose was added and incubated for 30 minutes at room temperature. Flow cytometric cell cycle analysis was then performed.

## 7.2 RESULTS

### 7.2.1 TIME LINE OF EVENTS FOR ACTIVATION OF OVAL CELL PROLIFERATION

To activate oval cell proliferation in the liver, certain events must occur. Figure 6 is an outline that represents the events and times involved in the process of the activation of oval cell proliferation. In control animals, a placebo was inserted in place of 2-AAF. Oval cells can be seen as early as day 5 posthepatic injury and can still be detected as late as day 42 postinjury. Figure 7A-B shows the histological changes in liver sections from rats exposed to 2-AAF for 7 days, followed by CCl<sub>4</sub> exposure and sacrificed 11 days posthepatic injury. With the 2-AAF/CCl<sub>4</sub> protocol, massive oval cell proliferation was seen after day 9. The sections were also stained with antibodies for specific oval cell markers (e.g., CK-19 and GGT), and the oval cells generated from the 2-AAF/CCl<sub>4</sub> protocol were positive for these markers. The major peak of BrdU incorporation for oval cells occurred at day 9 posthepatic injury, with a drastic drop-off in BrdU incorporation on subsequent days. These results can be seen in Figure 8A-D, which shows livers sections from rats sacrificed on day 9 (Figure 8A), day 11 (Figure 8B), and day 13 (Figure 8C) post-CCl<sub>4</sub> exposure. The same type of proliferation pattern was also seen in the 2-AAF/Phx model.

### 7.2.2. IMMUNOHISTOCHEMISTRY FOR THY-1 ON ACTIVATED HEPATIC OVAL CELLS

To determine whether Thy-1 and OC.2 antigens are expressed only by oval cells, normal rat liver was first examined. Figure 4 represents frozen sections  
5 obtained from normal rat liver. These sections were stained with Thy-1.1 antibody (Figure 9A) or OC.2 antibody (Figure 9B). There appears to be no portion of the liver expressing the Thy-1 antigen. Figure 9B represents OC.2 staining. The ductular cells appear to be positive with little to no staining elsewhere in the liver. Our results are in agreement with Faris et al. (1991, Cancer Res 51:1308-1317), who previously  
10 reported this pattern of staining for OC.2 in normal liver.

To test for Thy-1 expression on oval cells, frozen sections of livers with proliferating oval cells were used. In addition to Thy-1 expression, CK-19 and OC.2 expression was also examined. Figure 10 represents frozen liver sections  
15 obtained from rats on the 2-AAF/ $\text{CCl}_4$  protocol at day 21 after liver injury. These sections were stained for CK-19 (Figure 10A and 10D), OC.2 (Figure 10B and 10E), and Thy-1 (Figure 10C and 10F). Staining by all three antibodies on serial sections showed similar patterns, with all staining located in the periportal region and spreading outward; the pericentral region is devoid of staining. At higher magnification (Figure 10 D-F), the reactivity of the antibodies was primarily to the  
20 oval cells (arrows), or what has been termed in the art as transitional cells. Thy-1 antibody stained the ductular epithelium, which showed negative staining. This is also evident in Figure 11.

The possibility that cells staining positive for Thy-1 may be inflammatory cells in response to the  $\text{CCl}_4$  exposure was ruled out. In the 2-AAF/PHx  
25 model, however, there is presumably no influx of inflammatory cells. Figure 11 shows sections from livers with oval cells, in which the hepatic injury was a PHx of two thirds (day 13 2-AAF/PHx). Again, as shown in Figure 10, the pattern of staining in the periportal region of the lobule and expanding outward was similar to the staining pattern seen in livers from a 2-AAF/PHx model (Figure 11A-C). At a higher  
30 magnification (Figure 11D-F), the staining appeared localized to the oval cell population. These cells were positive for all three markers (CK-19 (Figure 11A and

11D); OC.2 (Figure 11B and 11E); Thy-1 (Figure 11C-11F)). Clearly, with both models of hepatic injury (2-AAF/CCl<sub>4</sub>, 2-AAF/PHx), the pattern of staining for CK-19, OC.2, and Thy-1 was the same within the same population of cells. It should also be noted that frozen sections from both 2-AAF/CCl<sub>4</sub> and 2-AAF/PHx were double-labeled with Thy-1 FITC and OC.2-Texas Red, and the same staining pattern was seen.

### 7.2.3. FACS ANALYSIS AND SORTING

#### FOR HOC USING THE THY-1 MARKER

As shown above, using immunohistochemistry, oval cells express Thy1.1 antigen. The possibility of obtaining a distinct Thy-1<sup>+</sup> population by cell sorting was next tested. Figure 12 represents FACS analysis of Thy-1<sup>+</sup> and Thy-1<sup>-</sup> sorted oval cells obtained from day 12 liver on the 2-AAF/CCl<sub>4</sub> protocol. Both the forward-/side-scatter plots and histograms are shown. Figure 12A (right gate) is that of Thy-1<sup>+</sup> cells, and Figure 12B (left gate) is Thy-1<sup>-</sup> cells. The Thy1.1<sup>+</sup> population was sorted to 97% purity and the Thy1.1<sup>-</sup> cells to a 99% purity. In each experiment, only a portion of the NPC fraction was sorted (60 to 80 x 10<sup>6</sup> cells) and typical yields of Thy-1<sup>+</sup> cells obtained were 15 to 20 x 10<sup>6</sup> cells. These two populations of sorted cells were 95% viable by Trypan blue exclusion. The Thy1.1<sup>+</sup> sorted cells were further examined to determine what stage of the cell cycle they were in. Oval cells were stained with propidium iodide and analyzed. Cell cycle analysis in Figure 12C shows a majority (>90%) of the Thy1.1<sup>+</sup> cells were in the G0/G1 stage of the cell cycle. Figure 12D shows that ≈90% of those cells in the G0/G1 stage of the cell cycle were Thy-1<sup>+</sup>.

Immunohistochemical staining for BrdU and PCNA (Figure 13A and 13B, respectively) also revealed that the oval cells were not in the cell cycle at the stage of the model used for this study. Only a few cells were positively stained for either marker, representing the second peak in the propidium iodide analysis (Figure 13C and 13D). In a separate study, it was shown that there was only one peak (day 9) of BrdU incorporation for oval cells (2-AAF/CCl<sub>4</sub> protocol), and the days following the peak showed very little incorporation. Others have shown that oval cell

proliferation lasts much longer, and perhaps the differences can be explained by the differences in the protocols. The data shown here were from experiments using the 2-AAF/CCl<sub>4</sub> protocol.

#### 7.2.4. IMMUNOHISTOCHEMISTRY ON THY-1<sup>+</sup> SORTED HOC

5            Having obtained Thy1.1<sup>+</sup> and Thy-1<sup>-</sup> populations, the sorted populations were further characterized to determine if the traditional oval cell markers were expressed. Thy-1<sup>+</sup> and Thy-1<sup>-</sup> cells were cytocentrifugated onto slides. Figures 14 and 15 represent Thy-1<sup>+</sup> and Thy-1<sup>-</sup> cells, respectively. Figure 14A is a hematoxylin-eosin stain, and Figure 14B is a representative of a negative control in which the primary antibody was omitted. For all antibodies, the appropriate negative controls were performed, either by omitting the primary antibody or by blocking with an appropriate nonimmune serum. Figure 14C-14F is of sorted Thy-1<sup>+</sup> cells stained with  $\alpha$ -fetoprotein (AFP), CK-19, GGT, and OV6, respectively. To show that the cells of interest (Thy-1<sup>+</sup> cells) are not Ito cells, staining for desmin, an Ito cell-specific marker, was performed. These data are shown in Fig. 14G. The Thy-1<sup>-</sup> cells were negative for desmin, which indicated that these cells were Ito cells. Also, desmin staining was performed on liver sections obtained from both 2-AAF/CCl<sub>4</sub>- and 2-AAF/PHx-treated animals at various time points, and the oval cells were also negative. The results shown in Fig. 14H are of double-stained oval cells showing 10 Thy-1-FITC (green) and AFP-Texas Red (red). In regions of the cell in which the antibody binding is in close proximity to each other, the two fluorochrome wavelengths mix, and the resulting fluorescence is yellow. The presence of dual markers (yellow) was evident in most cells shown. In addition, both Thy1 (green) and AFP (red) were seen as distinct colors in separate cellular domains on the same cell. 15

20            To show that the Thy-1<sup>+</sup> staining was specific for oval cells, Thy-1<sup>-</sup> cells were also subjected to the same immunostaining described above. Figure 15 represents these Thy-1<sup>-</sup> cells; Figure 15A is a representative hematoxylin-eosin stain of Thy-1<sup>-</sup> cells. Figure 15B and 15D are cells stained for AFP and GGT, respectively. Their corresponding negative controls are shown in Fig. 15C and 15D, respectively. 25

30            In both cases, the Thy-1<sup>-</sup> populations were devoid of any staining for these two

traditional oval cell markers. The specificity for Thy-1 binding appeared to be only specific to oval cells, as seen in Figures 14 and 15.

5 The present invention is not to be limited in scope by the specific embodiments described herein which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such  
10 modifications are intended to fall within the scope of the claims. Various publications are cited herein, the contents of which are hereby incorporated, by reference, in their entireties.

CLAIMS

1. A method for stimulating liver regeneration in a subject having a liver disorder comprising administering of bone marrow cells to said subject in an amount sufficient to result in the production of hepatocytes, bile ductal cells and/or oval cells.  
5
2. The method of Claim 1 wherein the bone marrow cells are injected.
3. The method of Claim 1 wherein the bone marrow cells are transplanted into the liver.
4. The method of Claim 1 wherein the bone marrow cells are genetically engineered to express a functionally active protein.  
10
5. The method of Claim 1 wherein the bone marrow cells are on a support matrix.
6. A method for stimulating liver regeneration in a subject having a liver disorder comprising the administration of enriched oval cells to a subject in an amount sufficient to result in the production of hepatocytes, bile ductal cells and/or oval cells.  
15
7. The method of Claim 6 wherein the oval cells are injected.
8. The method of Claim 6 wherein the oval cells are transplanted into the liver.  
20
9. The method of Claim 6 wherein the oval cells are genetically engineered to express a functionally active protein.

10. The method of Claim 6 wherein the oval cells are on a support matrix.
11. The method of Claim 1 or 6 wherein the dose of cells is between  $10^5$ - $10^6$ .
- 5 12. The method of Claim 1 or 6 wherein the dose of cells is between  $10^6$ - $10^8$ .
13. The method of Claim 1 wherein the bone marrow cells are contacted with a growth factor prior to administration.
- 10 14. The method of Claim 6 wherein the oval cells are contacted with a growth factor prior to administration.
- 15 15. A method for stimulating pancreatic regeneration in a subject having a pancreatic disorder comprising administering of bone marrow cells to said subject in an amount sufficient to result in the production of pancreatic cells.
16. The method of Claim 15 wherein the bone marrow cells are injected.
17. The method of Claim 15 wherein the bone marrow cells are transplanted into the pancreas.
- 20 18. The method of Claim 15 wherein the bone marrow cells are genetically engineered to express a functionally active protein.
19. The method of Claim 15 wherein the bone marrow cells are on a support matrix.

20. A method for enriching for oval cells comprising:
- (a) disaggregating liver tissue to form a single cell suspension of hepatic cells;
  - (b) purification from the single cell suspension of Thy1.1 positive hepatic cells from Thy1.1 negative hepatic cells;
- 5 wherein the Thy1.1 positive cells comprise the oval cells.
21. The method of Claim 20 wherein Thy1.1 positive hepatic cells are purified from Thy1.1 negative cells using a Thy-1 specific antibody.
22. A composition comprising an enriched population of oval cells in a physiologically acceptable carrier.
- 10
23. A composition of matter comprising an enriched population of oval cells attached to a matrix.
24. The composition of matter of Claim 23 further comprising a growth factor associated with the matrix.



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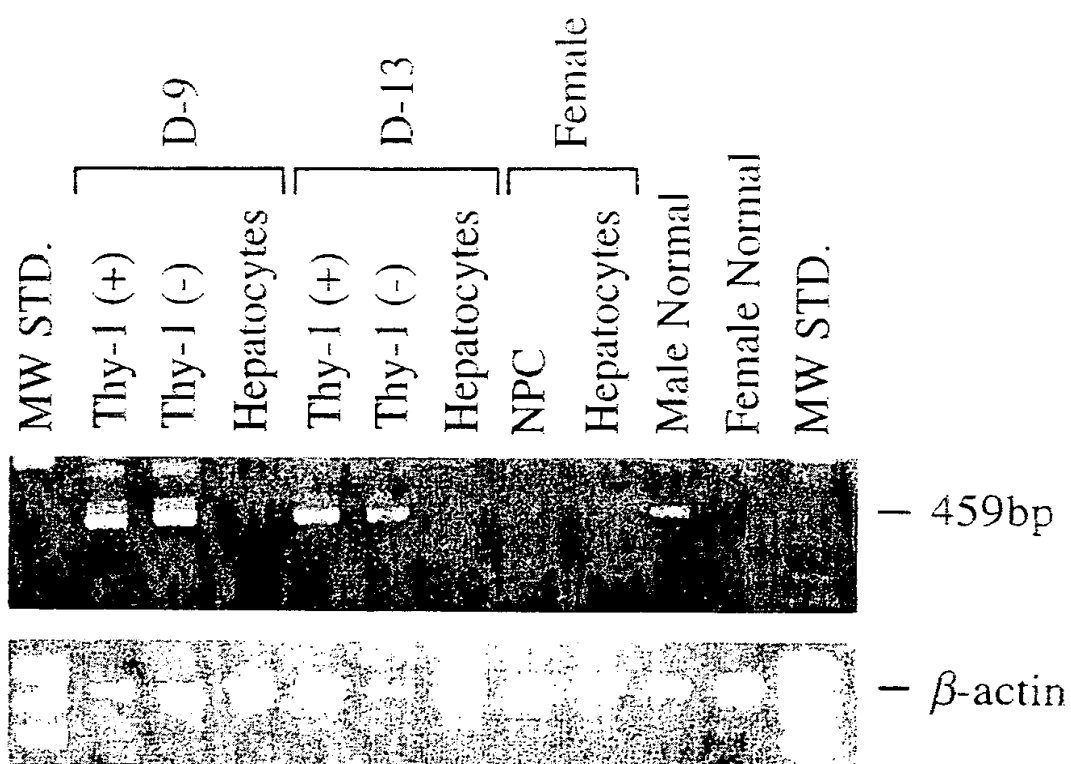


FIG. 1

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FIG.2A

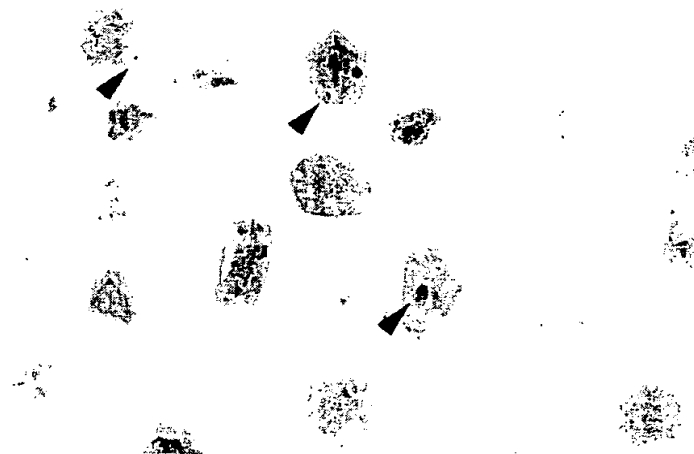


FIG.2B

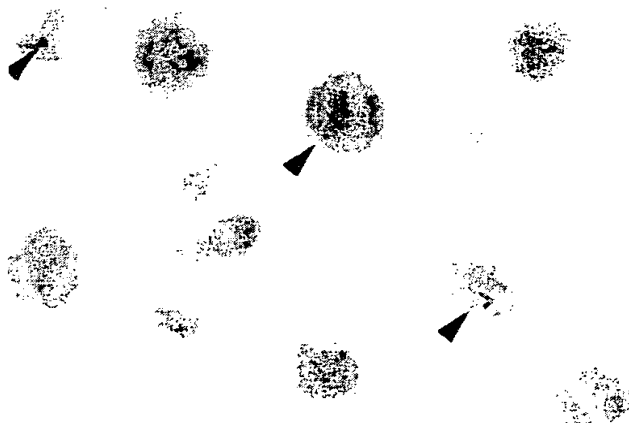


FIG.2C

FIG.3B



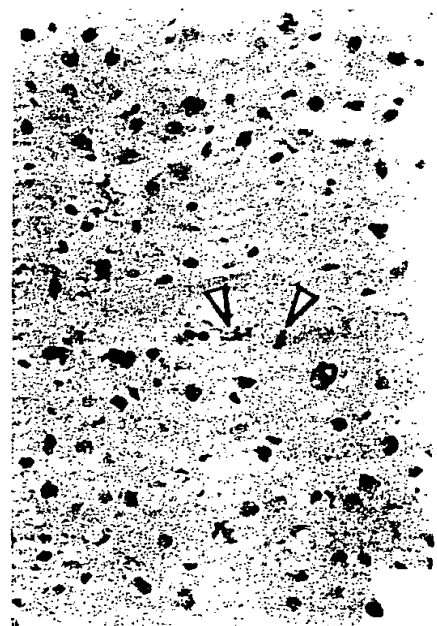
FIG.3A



FIG.3D



FIG.3C



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FIG.3F

FIG.3E

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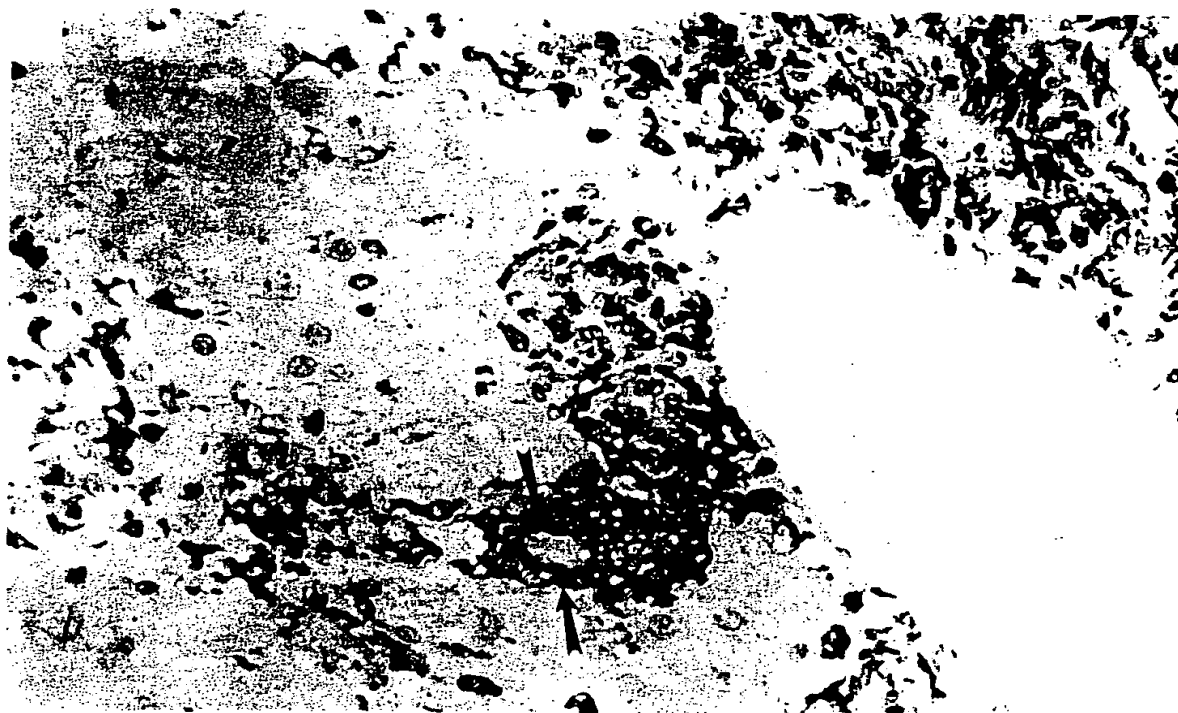


FIG.4A

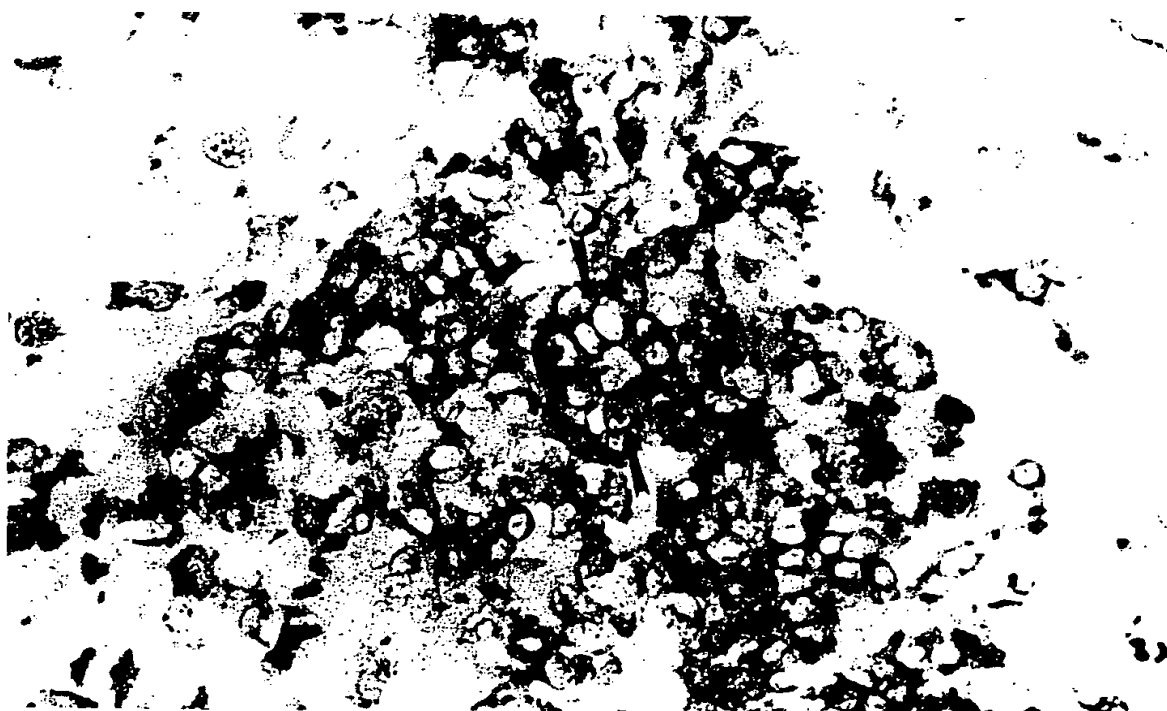


FIG.4B

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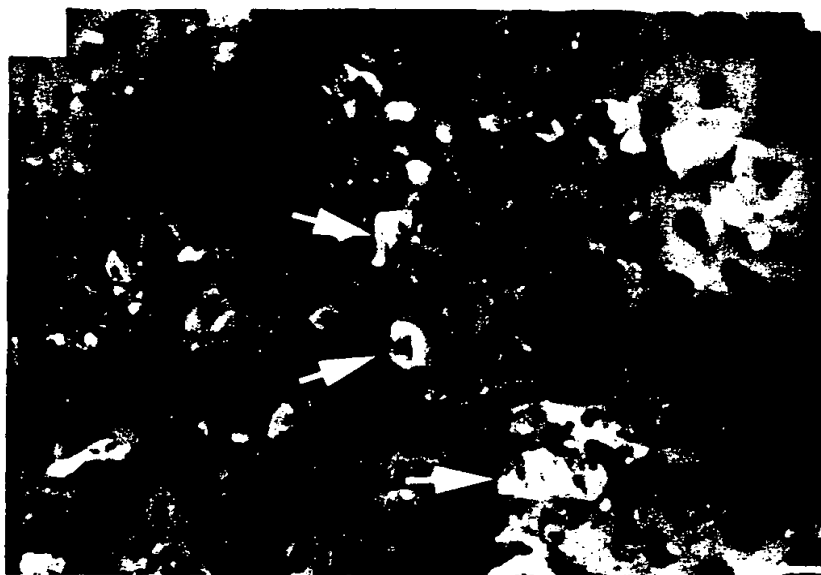


FIG. 5A



FIG. 5B



FIG. 5C

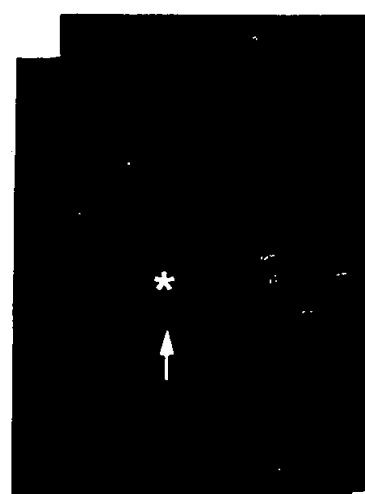


FIG. 5D

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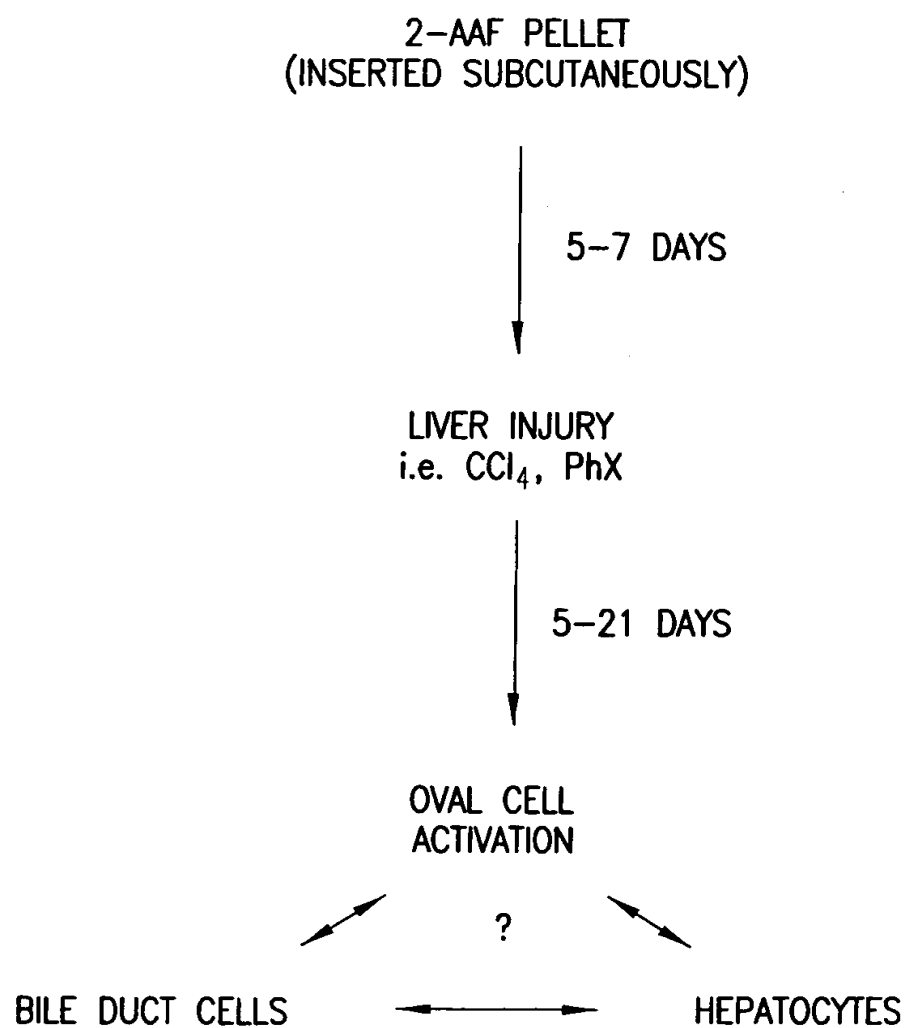


FIG.6

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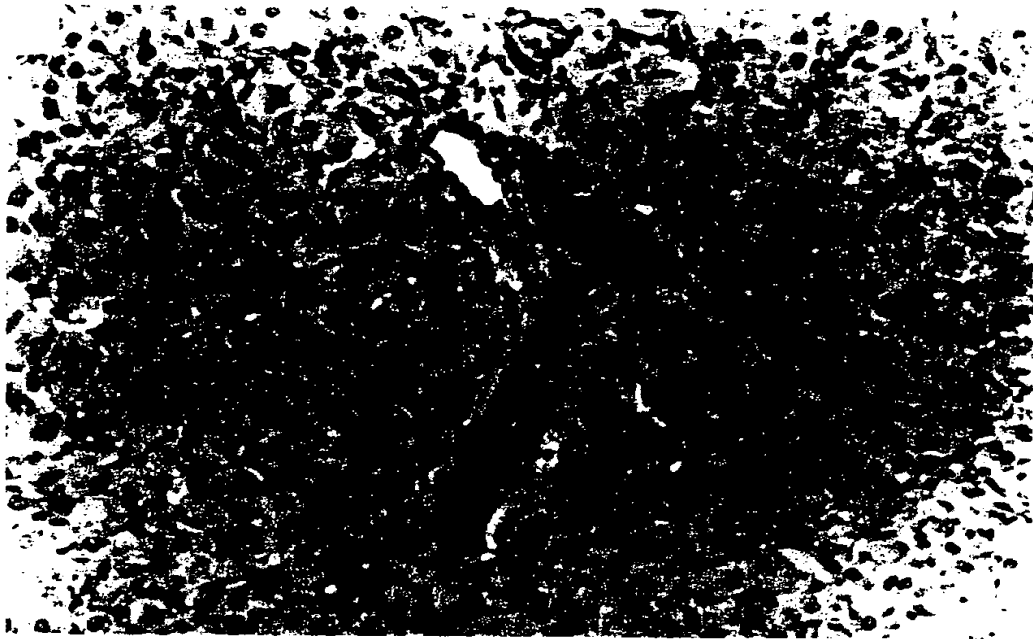


FIG.7A

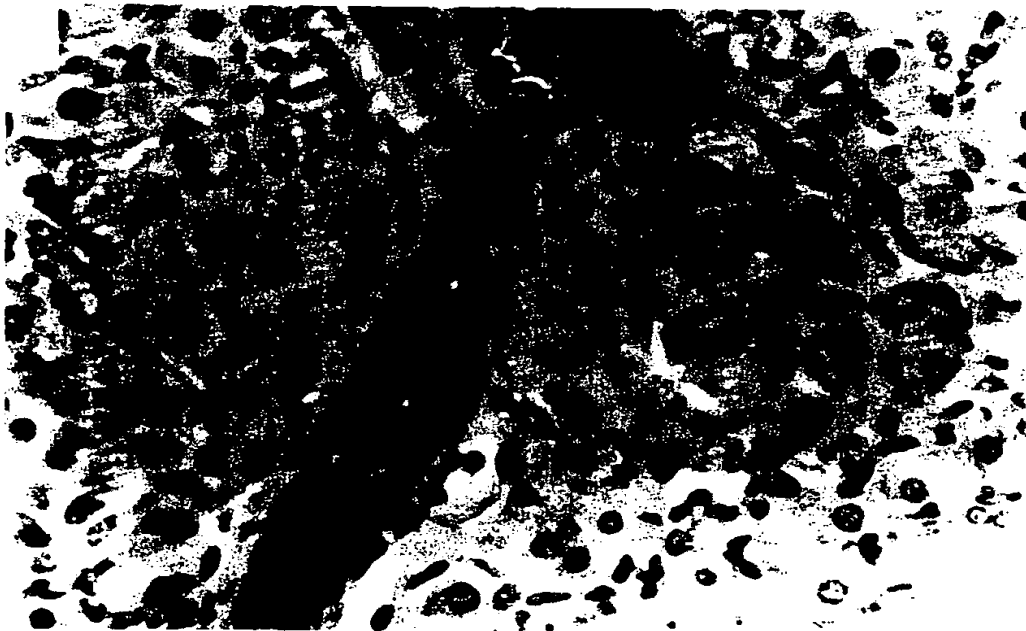


FIG.7B



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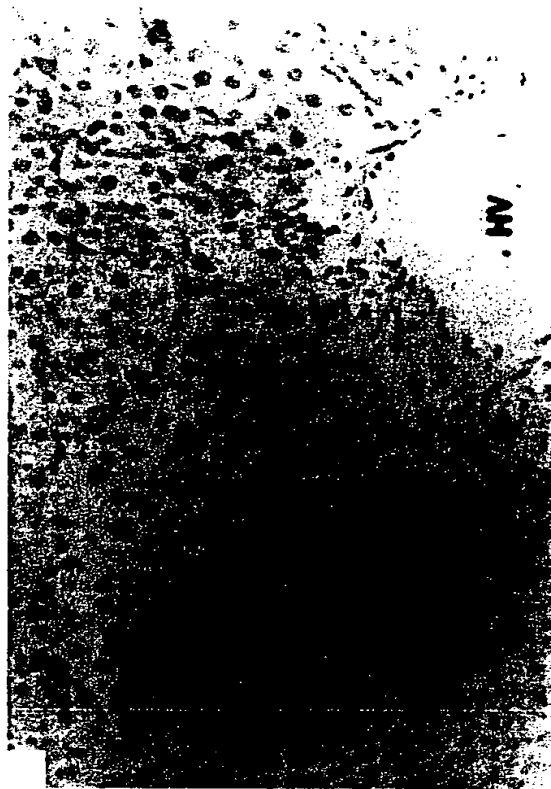


FIG.8B



FIG.8A

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FIG. 8D

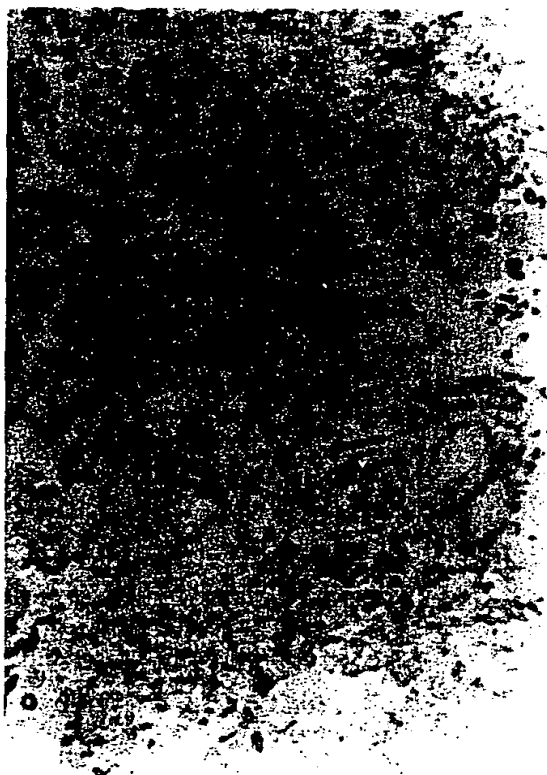


FIG. 8C

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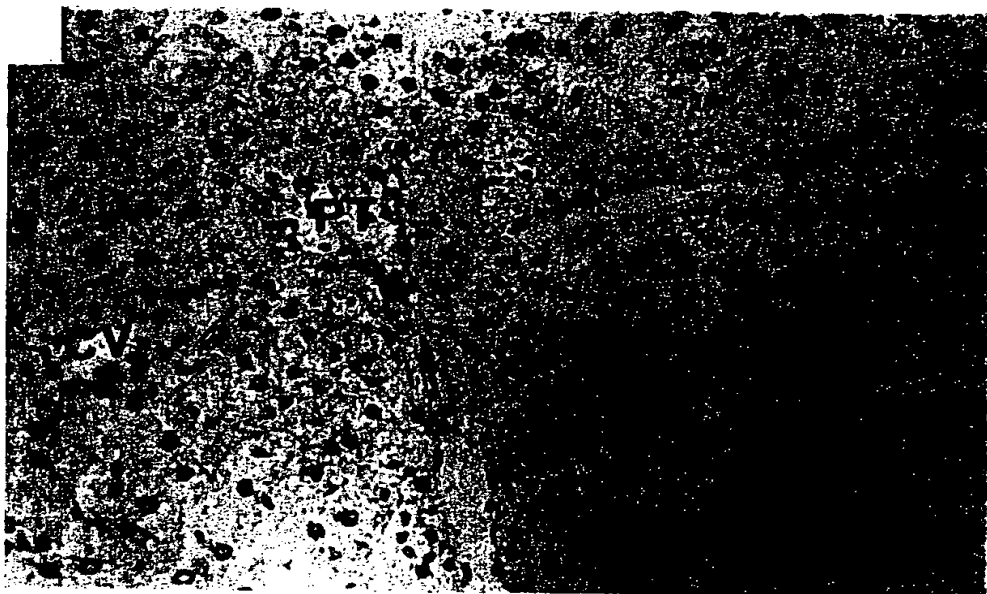


FIG.9A

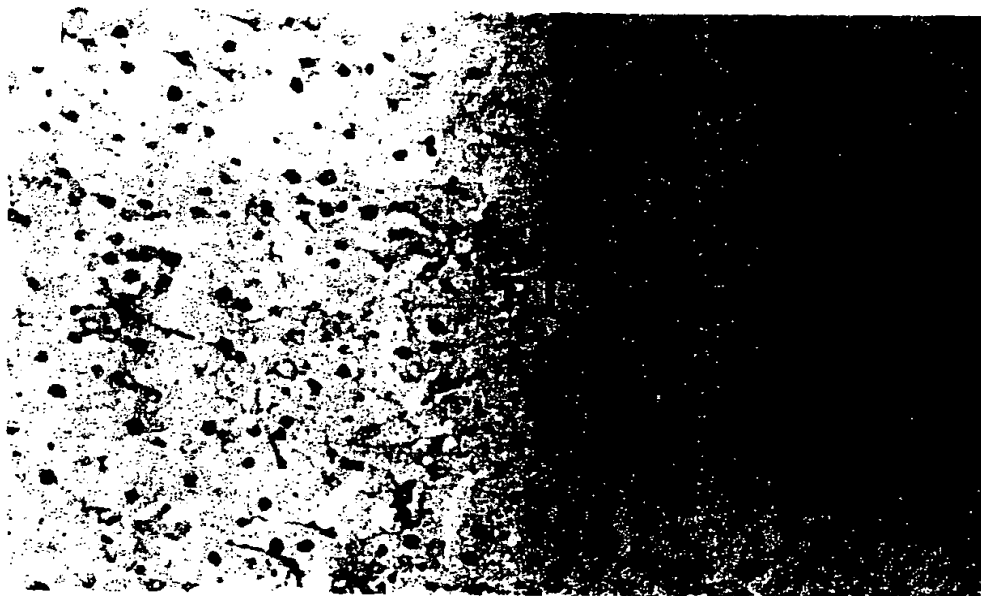


FIG.9B

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FIG. 10A

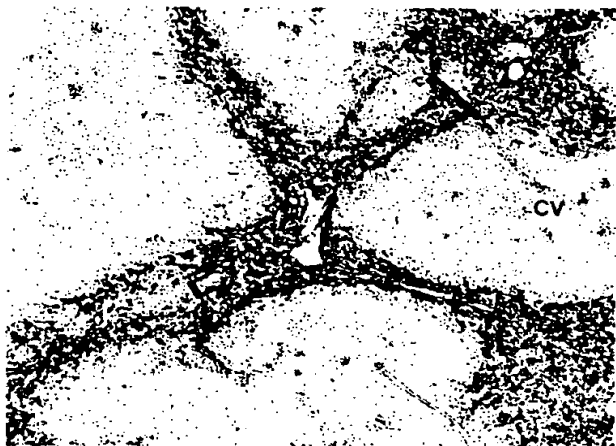


FIG. 10B

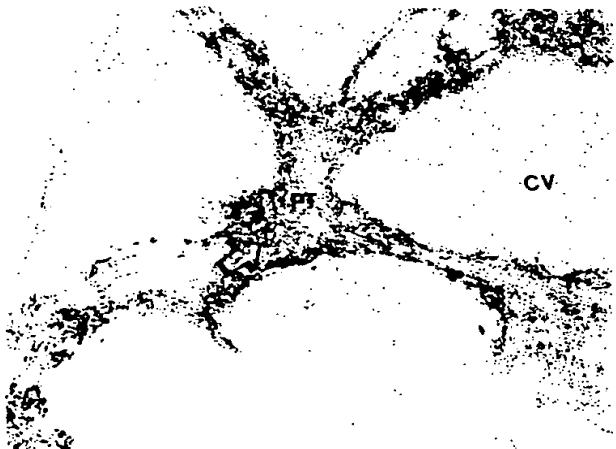


FIG. 10C

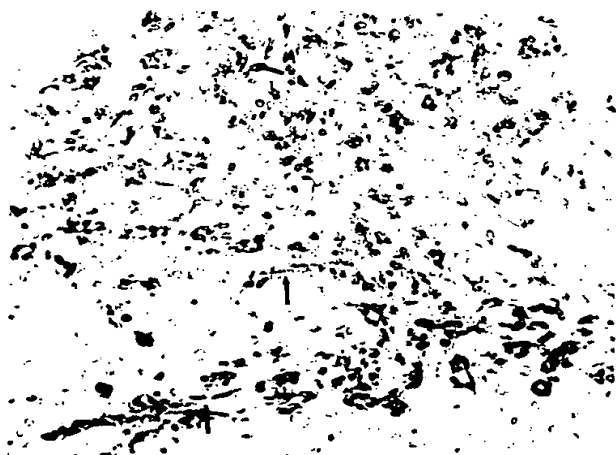


FIG.10D

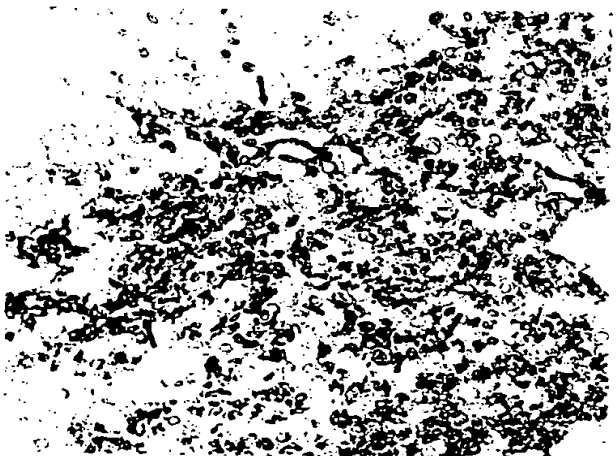


FIG.10E

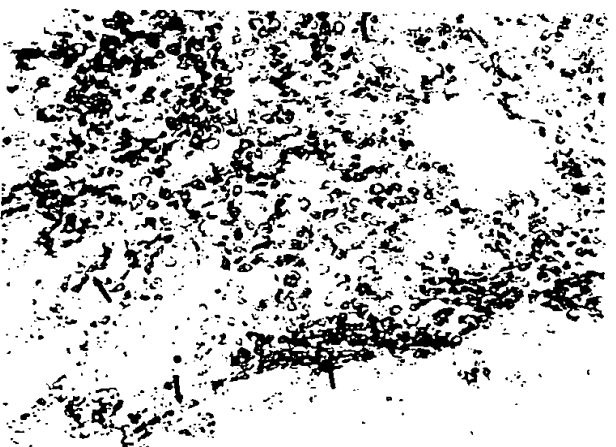


FIG.10F

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FIG. 11A

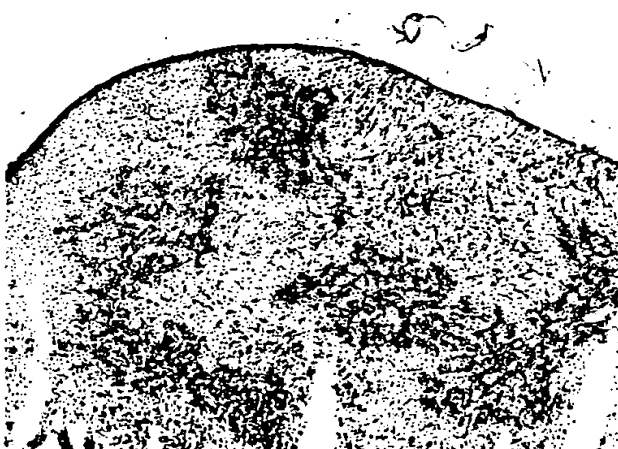


FIG. 11B

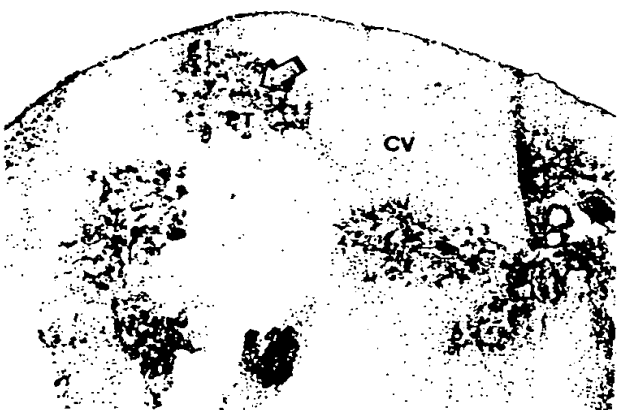


FIG. 11C

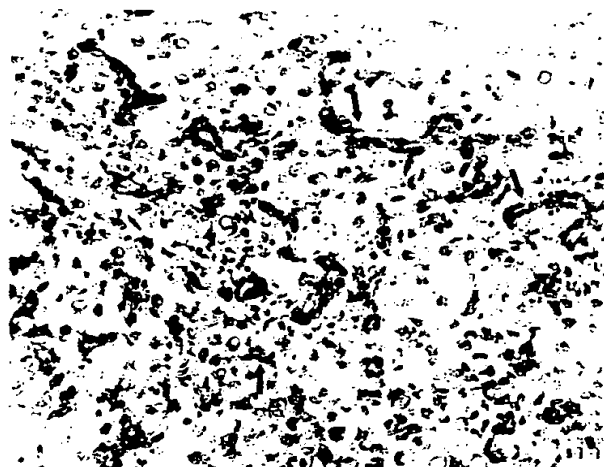


FIG. 11D

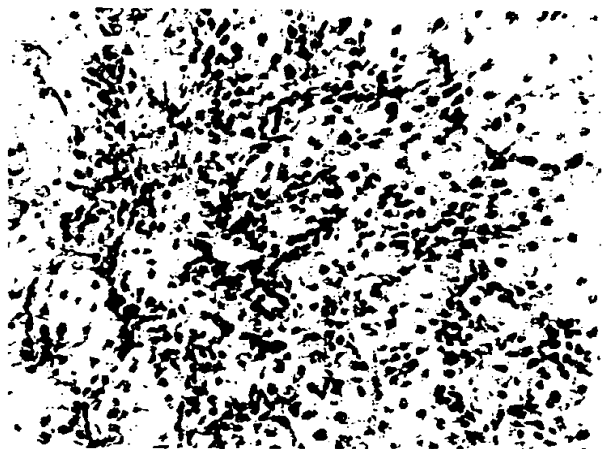


FIG. 11E

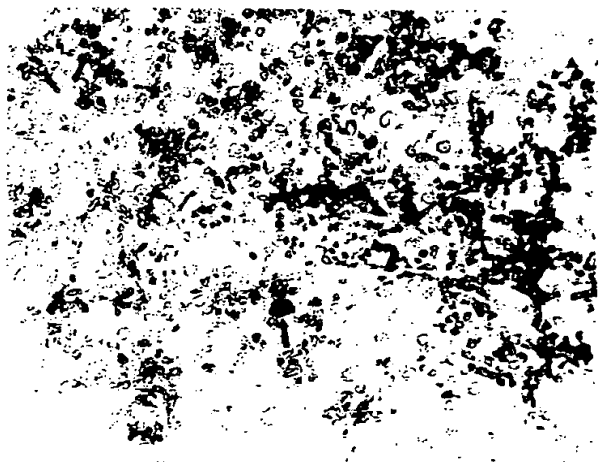


FIG. 11F

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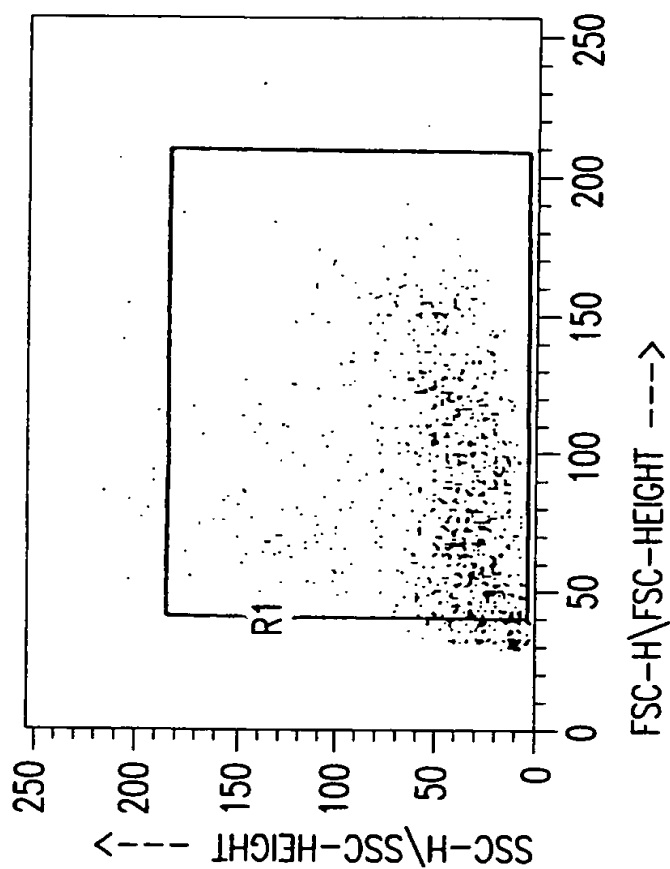


FIG.12A

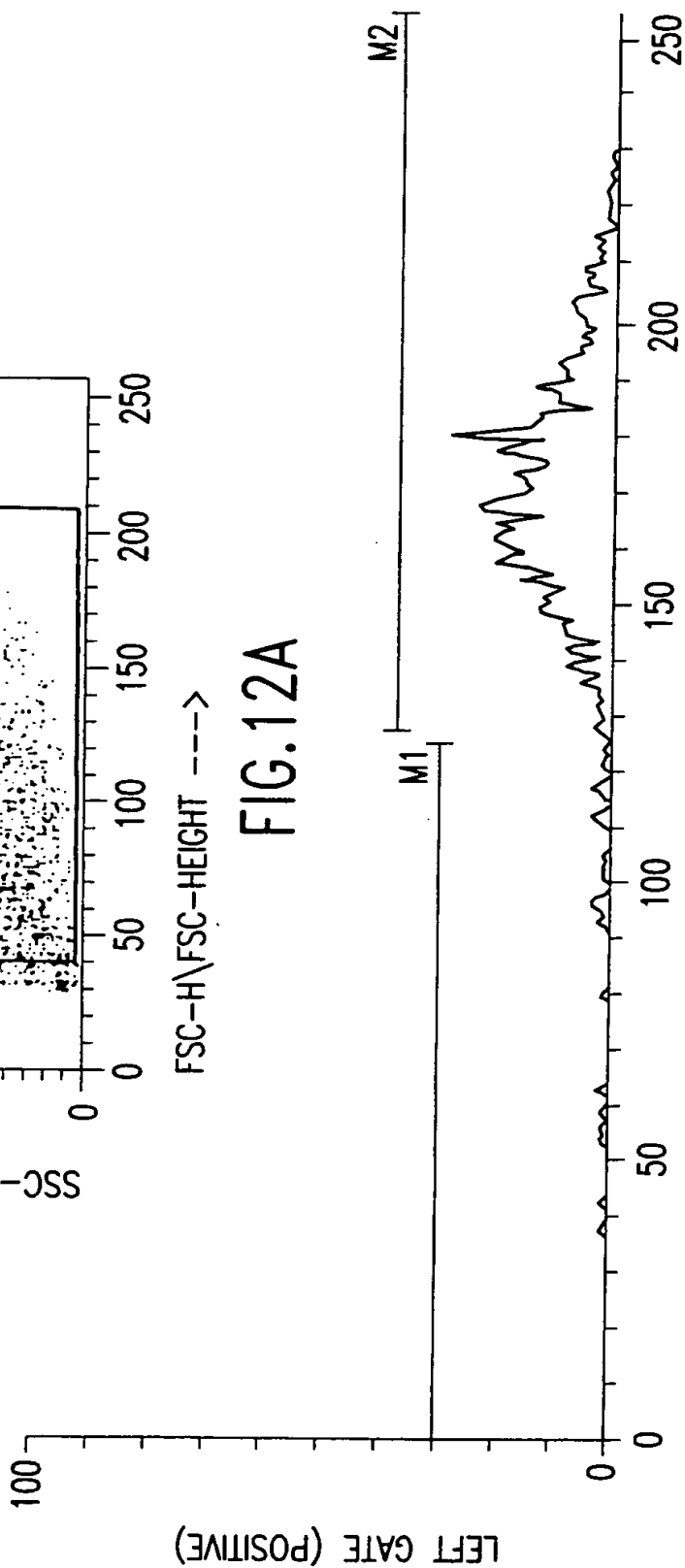


FIG.12A-1



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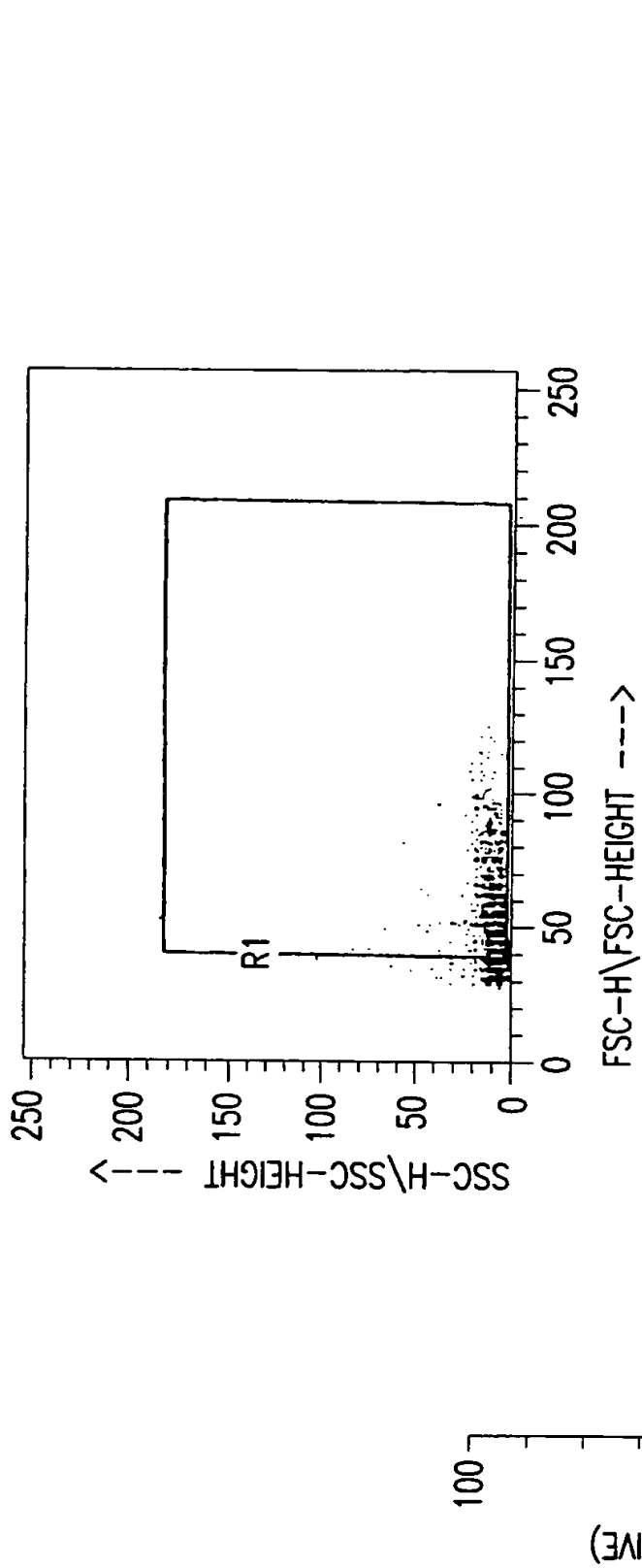


FIG.12B

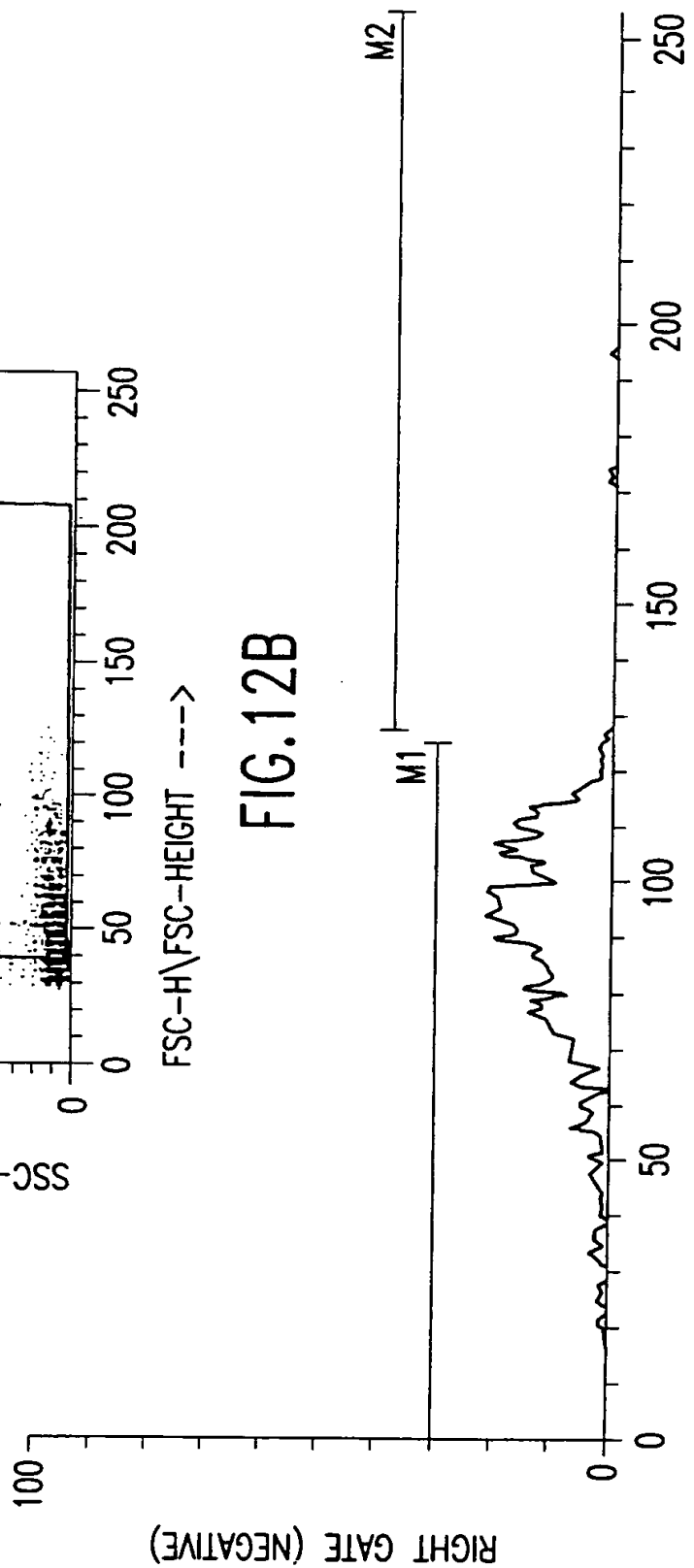


FIG.12B-1

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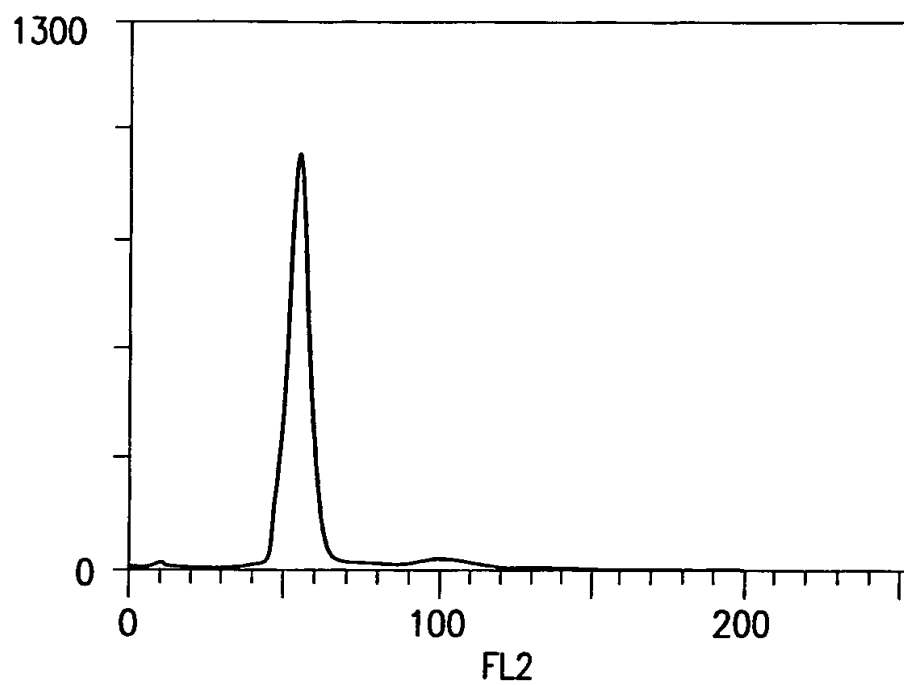


FIG.12C

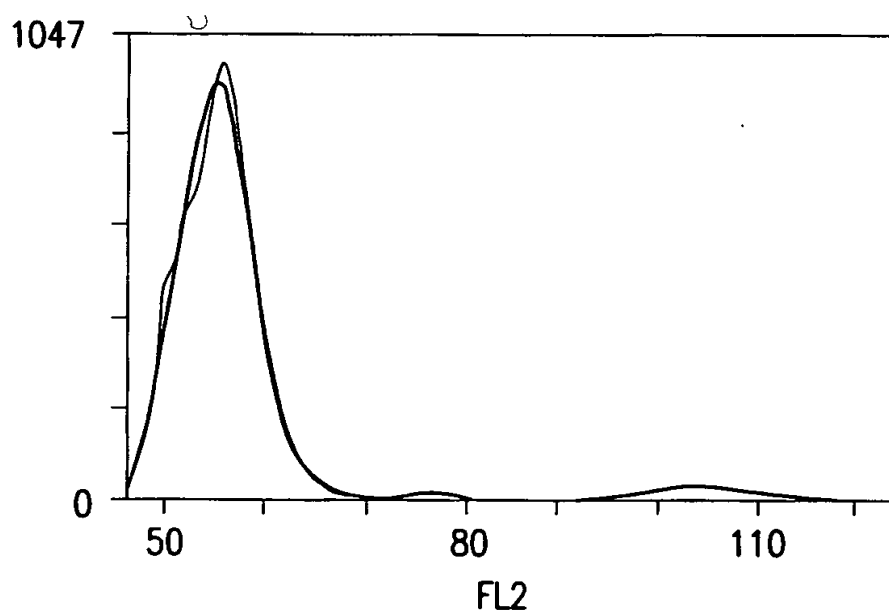


FIG.12D

FIG.13B

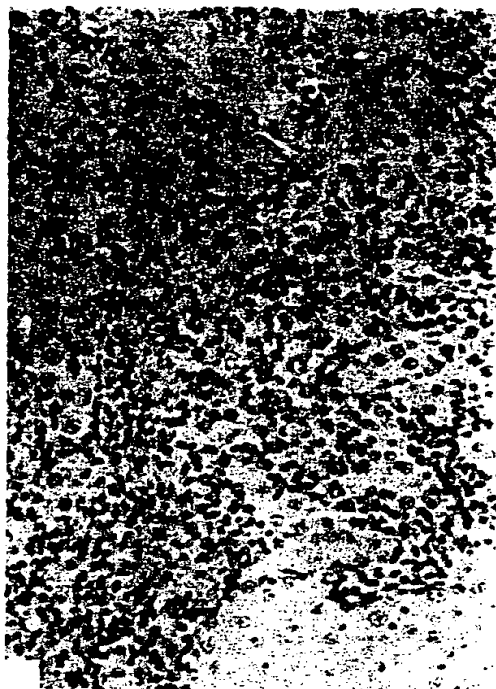


FIG.13D



FIG.13A



FIG.13C

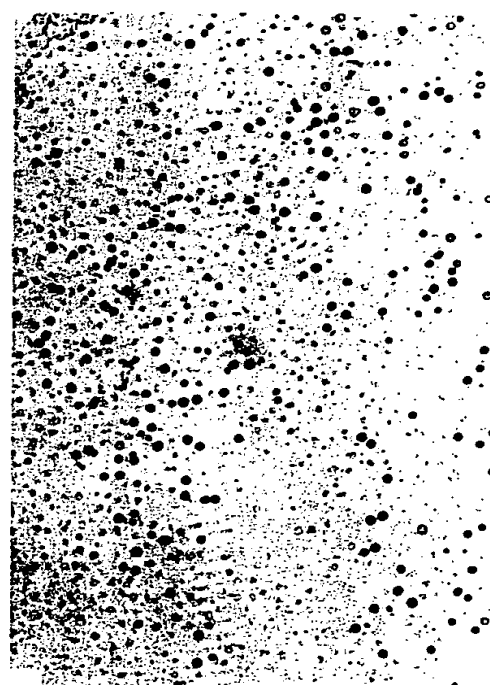


FIG.14B



FIG.14D



FIG.14A



FIG.14C

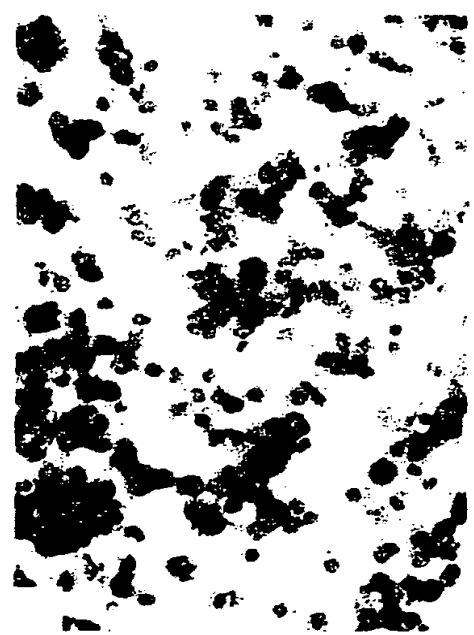


FIG. 14F



FIG. 14H



FIG. 14E



FIG. 14G



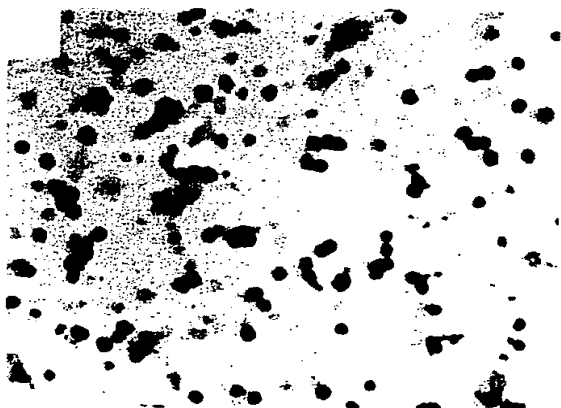


FIG. 15A

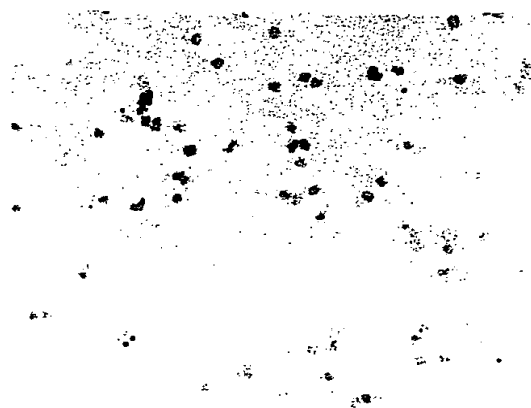


FIG. 15B

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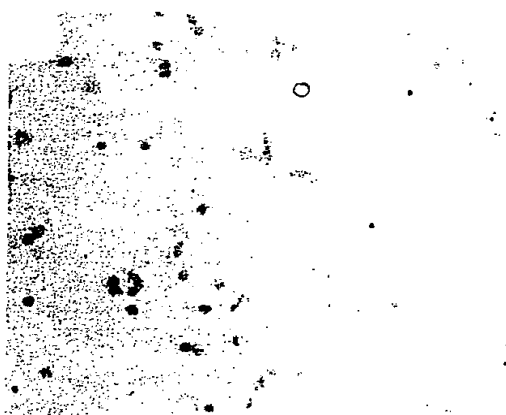


FIG. 15C



FIG. 15D

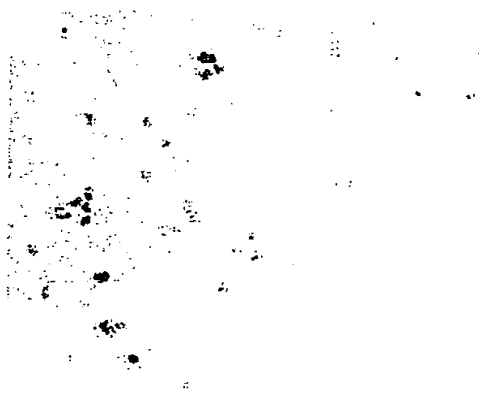


FIG. 15E

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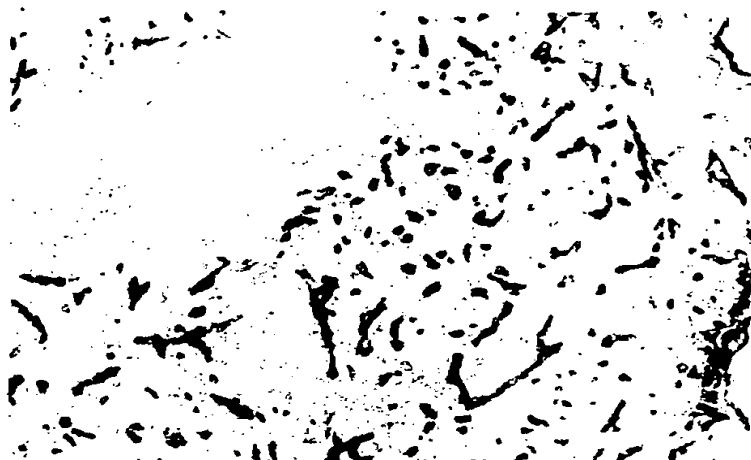


FIG.16A



FIG.16B



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FIG.16C



FIG.16D



FIG.17A

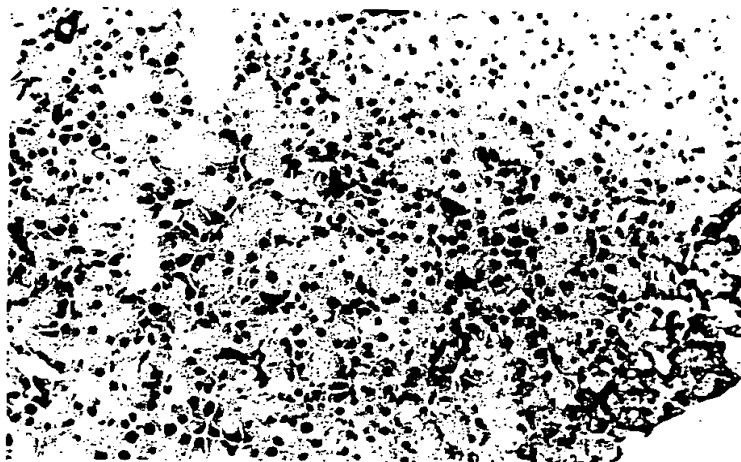


FIG.17B

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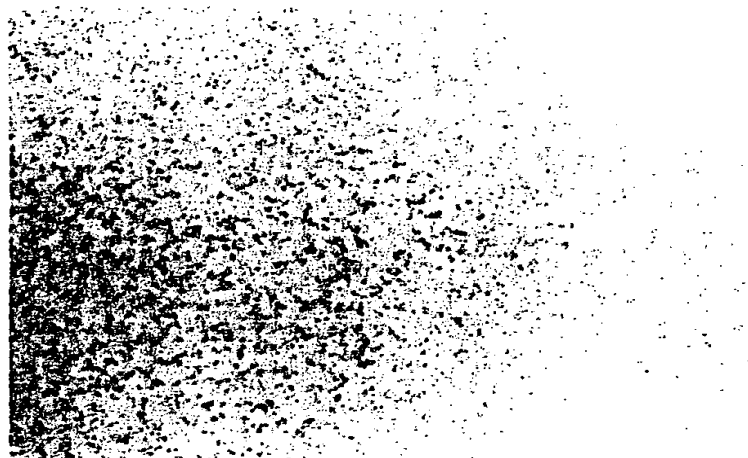


FIG.17C

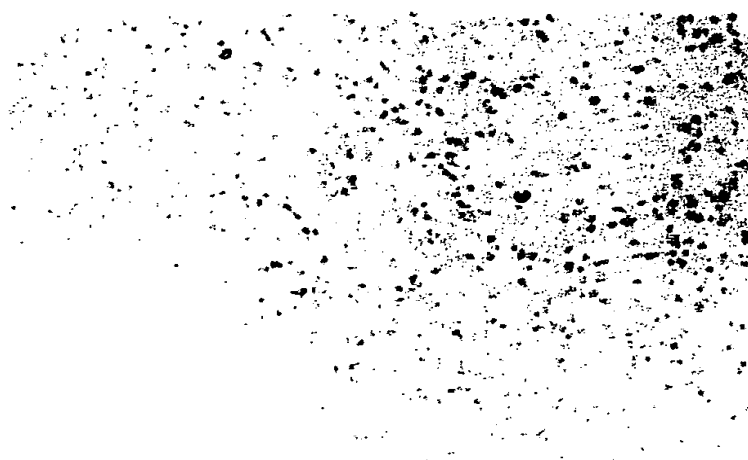


FIG.17D

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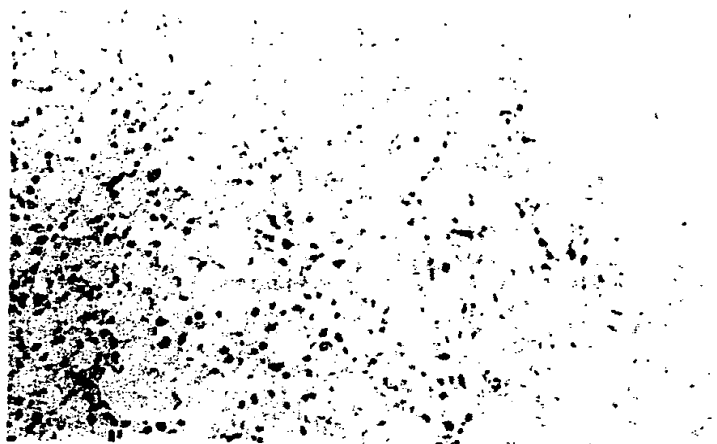


FIG.17E

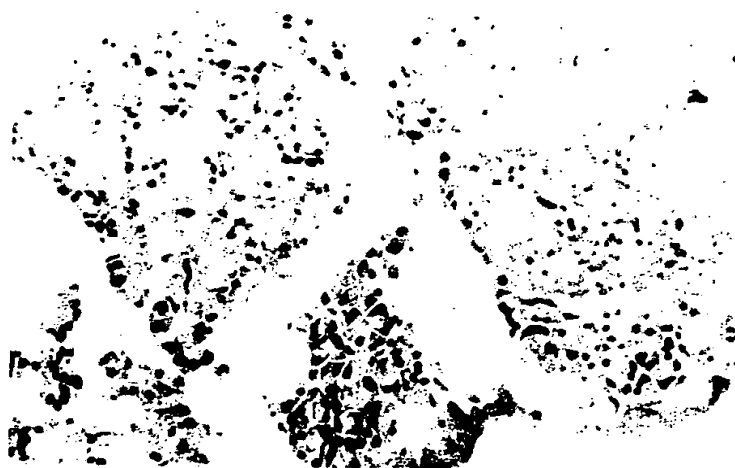


FIG.17F

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/04744

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K35/28 A61K35/407 A61P1/16 A61P1/18 //C12N5/06,  
C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE, CANCERLIT, CHEM ABS Data, EMBASE, SCISEARCH, EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 789 246 A (REID LOLA M ET AL) 4 August 1998 (1998-08-04) the whole document	1-5, 11-13
X	PETERSEN BRYON E ET AL: "Thy-1+ sorted hepatic oval cells in culture maintain oval cell phenotypic markers." FASEB JOURNAL, vol. 12, no. 4, 17 March 1998 (1998-03-17), page A468 XP002147734 ISSN: 0892-6638 the whole document	20-23

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

18 September 2000

Date of mailing of the international search report

29/09/2000

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/04744

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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